T Cell Receptor Display

The present invention relates to proteinaceous particles, for example phage or ribosome particles, displaying T cell receptors (TCRs), and diverse libraries thereof.

Background to the Invention

Native TCRs

As is described in, for example, WO 99/60120 TCRs mediate the recognition of specific Major Histocompatibility Complex (MHC)-peptide complexes by T cells and, as such, are essential to the functioning of the cellular arm of the immune system.

Antibodies and TCRs are the only two types of molecules which recognise antigens in a specific manner, and thus the TCR is the only receptor for particular peptide antigens presented in MHC, the alien peptide often being the only sign of an abnormality within a cell. T cell recognition occurs when a T-cell and an antigen presenting cell (APC) are in direct physical contact, and is initiated by ligation of antigen-specific TCRs with pMHC complexes.

The native TCR is a heterodimeric cell surface protein of the immunoglobulin superfamily which is associated with invariant proteins of the CD3 complex involved in mediating signal transduction. TCRs exist in $\alpha\beta$ and $\gamma\delta$ forms, which are structurally similar but have quite distinct anatomical locations and probably functions. The MHC class I and class II ligands are also immunoglobulin superfamily proteins but are specialised for antigen presentation, with a highly polymorphic peptide binding site which enables them to present a diverse array of short peptide fragments at the APC cell surface.

Two further classes of proteins are known to be capable of functioning as TCR ligands. (1) CD1 antigens are MHC class I-related molecules whose genes are located on a different chromosome from the classical MHC class I and class II antigens. CD1 molecules are capable of presenting peptide and non-peptide (eg lipid, glycolipid)

moieties to T cells in a manner analogous to conventional class I and class II-MHC-pep complexes. See, for example (Barclay et al, (1997)The Leucocyte Antigen Factsbook 2nd Edition, Acadmeic Press) and (Bauer (1997) Eur J Immunol **27** (6) 1366-1373)) (2) Bacterial superantigens are soluble toxins which are capable of binding both class II MHC molecules and a subset of TCRs.(Fraser (1989) Nature **339** 221-223) Many superantigens exhibit specificity for one or two Vbeta segments, whereas others exhibit more promiscuous binding. In any event, superantigens are capable of eliciting an enhanced immune response by virtue of their ability to stimulate subsets of T cells in a polyclonal fashion.

The extracellular portion of native heterodimeric αβ and γδ TCRs consist of two polypeptides each of which has a membrane-proximal constant domain, and a membrane-distal variable domain. Each of the constant and variable domains includes an intra-chain disulfide bond. The variable domains contain the highly polymorphic loops analogous to the complementarity determining regions (CDRs) of antibodies. CDR3 of αβ TCRs interact with the peptide presented by MHC, and CDRs 1 and 2 of αβ TCRs interact with the peptide and the MHC. The diversity of TCR sequences is generated via somatic rearrangement of linked variable (V), diversity (D), joining (J), and constant genes

Functional α and γ chain polypeptides are formed by rearranged V-J-C regions, whereas β and δ chains consist of V-D-J-C regions. The extracellular constant domain has a membrane proximal region and an immunoglobulin region. There are single α and δ chain constant domains, known as TRAC and TRDC respectively. The β chain constant domain is composed of one of two different β constant domains, known as TRBC1 and TRBC2 (IMGT nomenclature). There are four amino acid changes between these β constant domains, three of which are within the domains used to produce the single-chain TCRs displayed on phage particles of the present invention. These changes are all within exon 1 of TRBC1 and TRBC2: N_4K_5->K_4N_5 and F_{37}->Y (IMGT numbering, differences TRBC1->TRBC2), the final amino acid change

between the two TCR β chain constant regions being in exon 3 of TRBC1 and TRBC2: V_1->E. The constant γ domain is composed of one of either TRGC1, TRGC2(2x) or TRGC2(3x). The two TRGC2 constant domains differ only in the number of copies of the amino acids encoded by exon 2 of this gene that are present.

The extent of each of the TCR extracellular domains is somewhat variable. However, a person skilled in the art can readily determine the position of the domain boundaries using a reference such as The T Cell Receptor Facts Book, Lefranc & Lefranc, Publ. Academic Press 2001.

Recombinant TCRs

The production of recombinant TCRs is beneficial as these provide soluble TCR analogues suitable for the following purposes:

- Studying the TCR / ligand interactions (e.g. pMHC for αβ TCRs)
- Screening for inhibitors of TCR-associated interactions
- Providing the basis for potential therapeutics

A number of constructs have been devised to date for the production of recombinant TCRs. These constructs fall into two broad classes, single-chain TCRs and dimeric TCRs, the literature relevant to these constructs is summarised below.

Single-chain TCRs (scTCRs) are artificial constructs consisting of a single amino acid strand, which like native heterodimeric TCRs bind to MHC-peptide complexes. Unfortunately, attempts to produce functional alpha/beta analogue scTCRs by simply linking the alpha and beta chains such that both are expressed in a single open reading frame have been unsuccessful, presumably because of the natural instability of the alpha-beta soluble domain pairing.

Accordingly, special techniques using various truncations of either or both of the alpha and beta chains have been necessary for the production of scTCRs. These formats appear to be applicable only to a very limited range of scTCR sequences. Soo Hoo *et*

al (1992) PNAS. **89** (10): 4759-63 report the expression of a mouse TCR in single chain format from the 2C T cell clone using a truncated beta and alpha chain linked with a 25 amino acid linker and bacterial periplasmic expression (see also Schodin *et al* (1996) Mol. Immunol. **33 (9)**: 819-29). This design also forms the basis of the m6 single-chain TCR reported by Holler *et al* (2000) PNAS. **97** (10): 5387-92 which is derived from the 2C scTCR and binds to the same H2-Ld-restricted alloepitope. Shusta *et al* (2000) Nature Biotechnology **18**: 754-759 and US 6,423,538 report using a murine single-chain 2C TCR constructs in yeast display experiments, which produced mutated TCRs with, enhanced thermal stability and solubility. This report also demonstrated the ability of these displayed 2C TCRs to selectively bind cells expressing their cognate pMHC. Khandekar *et al* (1997) J. Biol. Chem. **272** (51): 32190-7 report a similar design for the murine D10 TCR, although this scTCR was fused to MBP and expressed in bacterial cytoplasm (see also Hare *et al* (1999) Nat. Struct. Biol. **6** (6): 574-81). Hilyard *et al* (1994) PNAS. **91** (19): 9057-61 report a human scTCR specific for influenza matrix protein-HLA-A2, using a Vα-linker-Vβ design and expressed in bacterial periplasm.

Chung *et al* (1994) PNAS. **91** (26) 12654-8 report the production of a human scTCR using a Vα-linker-Vβ-Cβ design and expression on the surface of a mammalian cell line. This report does not include any reference to peptide-HLA specific binding of the scTCR. Plaksin *et al* (1997) J. Immunol. **158** (5): 2218-27 report a similar Vα-linker-Vβ-Cβ design for producing a murine scTCR specific for an HIV gp120-H-2D^{d} epitope. This scTCR is expressed as bacterial inclusion bodies and refolded *in vitro*.

A number of papers describe the production of TCR heterodimers which include the native disulphide bridge which connects the respective subunits (Garboczi, *et al.*, (1996), Nature **384**(6605): 134-41; Garboczi, *et al.*, (1996), J Immunol **157**(12): 5403-10; Chang *et al.*, (1994), PNAS USA **91**: 11408-11412; Davodeau *et al.*, (1993), *J. Biol. Chem.* **268**(21): 15455-15460; Golden *et al.*, (1997), J. Imm. Meth. **206**: 163-169; US Patent No. 6080840). However, although such TCRs can be recognised by

TCR-specific antibodies, none were shown to recognise its native ligand at anything other than relatively high concentrations and/or were not stable.

In WO 99/60120, a soluble TCR is described which is correctly folded so that it is capable of recognising its native ligand, is stable over a period of time, and can be produced in reasonable quantities. This TCR comprises a TCR α or γ chain extracellular domain dimerised to a TCR β or δ chain extracellular domain respectively, by means of a pair of C-terminal dimerisation peptides, such as leucine zippers. This strategy of producing TCRs is generally applicable to all TCRs.

Reiter *et al*, Immunity, 1995, **2**:281-287, details the construction of a soluble molecule comprising disulphide-stabilised TCR α and β variable domains, one of which is linked to a truncated form of Pseudomonas exotoxin (PE38). One of the stated reasons for producing this molecule was to overcome the inherent instability of single-chain TCRs. The position of the novel disulphide bond in the TCR variable domains was identified via homology with the variable domains of antibodies, into which these have previously been introduced (for example see Brinkmann, *et al.* (1993), Proc. Natl. Acad. Sci. USA **90:** 7538-7542, and Reiter, *et al.* (1994) Biochemistry **33:** 5451-5459). However, as there is no such homology between antibody and TCR constant domains, such a technique could not be employed to identify appropriate sites for new inter-chain disulphide bonds between TCR constant domains.

As mentioned above Shusta *et al* (2000) Nature Biotechnology **18**: 754-759 report using single-chain 2 C TCR constructs in yeast display experiments. The principle of displaying scTCRs on phage particles has previously been discussed. For example, WO 99/19129 details the production of scTCRs, and summarise a potential method for the production of phage particles displaying scTCRs of the Vα-Linker-Vβ Cβ format. However, this application contains no exemplification demonstrating the production of said phage particles displaying TCR. The application does however refer to a co-pending application:

"The construction of DNA vectors including a DNA segment encoding a sc-TCR molecules fused to a bacteriophage coat protein (gene II or gene VIII) have been described in said pending U.S. application No. 08/813,781."

Furthermore, this application relies on the ability of anti-TCR antibodies or super-antigen MHC complexes to recognise the soluble, non-phage displayed, scTCRs produced to verify their correct conformation. Therefore, true peptide-MHC binding specificity of the scTCRs, in any format, is not conclusively demonstrated.
Finally, a further study (Onda *et al.*, (1995) Molecular Immunology **32** (17-18) 1387-1397) discloses the phage display of two murine TCR α chains in the absence of their respective β chains. This study demonstrated that phage particles displaying one of the TCR α chains (derived from the A1.1 murine hybridoma) bound preferentially to the same peptides immobilised in microtitre wells that the complete TCR would normally respond to when there were presented by the murine Class I MHC I-A^d.

Screening Use

A number of important cellular interactions and cell responses, including the TCR-mediated immune synapse, are controlled by contacts made between cell surface receptors and ligands presented on the surfaces of other cells. These types of specific molecular contacts are of crucial importance to the correct biochemical regulation in the human body and are therefore being studied intensely. In many cases, the objective of such studies is to devise a means of modulating cellular responses in order to prevent or combat disease.

Therefore, methods with which to identify compounds that bind with some degree of specificity to human receptor or ligand molecules are important as leads for the discovery and development of new disease therapeutics. In particular, compounds that interfere with certain receptor-ligand interactions have immediate potential as therapeutic agents or carriers.

Advances in combinatorial chemistry, enabling relatively easy and cost-efficient production of very large compound libraries have increased the scope for compound testing enormously. Now the limitations of screening programmes most often reside in the nature of the assays that can be employed, the production of suitable receptor and ligand molecules and how well these assays can be adapted to high throughput screening methods.

Display Methods

It is often desirable to present a given peptide or polypeptide on the surface of a proteinaceous particle. Such particles may serve as purification aids for the peptide or polypeptide (since the particles carrying the peptide or polypeptide may be separated from unwanted contaminants by sedimentation or other methods). They may also serve as particulate vaccines, the immune response to the surface displayed peptide or polypeptide being stimulated by the particulate presentation. Protein p24 of the yeast retrotransposon, and the hepatitis B surface coat protein are examples of proteins which self assemble into particles. Fusion of the peptide or polypeptide of interest to these particle-forming proteins is a recognised way of presenting the peptide or polypeptide on the surface of the resultant particles.

However, particle display methods have primarily been used to identify proteins with desirable properties such as enhanced expression yields, binding and/or stability characteristics. These methods involve creating a diverse pool or 'library' of proteins or polypeptides expressed on the surface of proteinaceous particles. These particles have two key features, firstly each particle presents a single variant protein or polypeptide, and secondly the genetic material encoding the expressed protein or polypeptide is associated with that of the particle. This library is then subjected to one or more rounds of selection. For example, this may consist of contacting a ligand with a particle-display library of mutated receptors and identifying which mutated receptors bind the ligand with the highest affinity. Once the selection process has been completed the receptor or receptors with the desired properties can be isolated, and their genetic material can be amplified in order to allow the receptors to be sequenced.

These display methods fall into two broad categories, *in-vitro* and *in-vivo* display.

All *in-vivo* display methods rely on a step in which the library, usually encoded in or with the genetic nucleic acid of a replicable particle such as a plasmid or phage replicon is transformed into cells to allow expression of the proteins or polypeptides. (Plückthun (2001) Adv Protein Chem **55** 367-403). There are a number of replicon/host systems that have proved suitable for *in-vivo* display of protein or polypeptides. These include the following

Phage / bacterial cells
plasmid / CHO cells
Vectors based on the yeast 2μm plasmid / yeast cells
bacculovirus / insect cells
plasmid / bacterial cells

In-vivo display methods include cell-surface display methods in which a plasmid is introduced into the host cell encoding a fusion protein consisting of the protein or polypeptide of interest fused to a cell surface protein or polypeptide. The expression of this fusion protein leads to the protein or polypeptide of interest being displayed on the surface of the cell. The cells displaying these proteins or polypeptides of interest can then be subjected to a selection process such as FACS and the plasmids obtained from the selected cell or cells can be isolated and sequenced. Cell surface display systems have been devised for mammalian cells (Higuschi (1997) J Immunol. Methods **202** 193-204), yeast cells (Shusta (1999) J Mol Biol **292** 949-956) and bacterial cells (Sameulson (2002) J. Biotechnol **96** (2) 129-154).

Numerous reviews of the various in-vivo display techniques have been published. For example, (Hudson (2002) Expert Opin Biol Ther (2001) **1** (5) 845-55) and (Schmitz (2000) **21** (Supp A) S106-S112).

In-vitro display methods are based on the use of ribosomes to translate libraries of mRNA into a diverse array of protein or polypeptide variants. The linkage between the proteins or polypeptides formed and the mRNA encoding these molecules is maintained by one of two methods. Conventional ribosome display utilises mRNA sequences that encode a short (typically 40-100 amino acid) linker sequence and the protein or polypeptide to be displayed. The linker sequence allow the displayed protein or polypeptide sufficient space to re-fold without being sterically hindered by the ribosme. The mRNA sequence lacks a 'stop' codon, this ensures that the expressed protein or polypeptide and the RNA remain attached to the ribosome particle. The related mRNA display method is based on the preparation of mRNA sequences encoding the protein or polypeptide of interest and DNA linkers carrying a puromycin moiety. As soon as the ribosome reaches the mRNA/DNA junction translation is stalled and the puromycin forms a covalent linkage to the ribosome. For a recent review of these two related *in-vitro* display methods see (Amstutz (2001) Curr Opin Biotechnol **12** 400-405).

Particularly preferred is the phage display technique which is based on the ability of bacteriophage particles to express a heterologous peptide or polypeptide fused to their surface proteins. (Smith (1985) Science **217** 1315-1317). The procedure is quite general, and well understood in the art for the display of polypeptide monomers. However, in the case of polypeptides that in their native form associate as dimers, only the phage display of antibodies appears to have been thoroughly investigated.

For monomeric polypeptide display there are two main procedures:
Firstly (Method A) by inserting into a vector (phagemid) DNA encoding the heterologous peptide or polypeptide fused to the DNA encoding a bacteriophage coat protein. The expression of phage particles displaying the heterologous peptide or polypeptide is then carried out by transfecting bacterial cells with the phagemid, and then infecting the transformed cells with a 'helper phage'. The helper phage acts as a source of the phage proteins not encoded by the phagemid required to produce a functional phage particle.

Secondly (Method B), by inserting DNA encoding the heterologous peptide or polypeptide into a complete phage genome fused to the DNA encoding a bacteriophage coat protein. The expression of phage particles displaying the heterologous peptide or polypeptide is then carried out by infecting bacterial cells with the phage genome. This method has the advantage of the first method of being a 'single-step' process. However, the size of the heterologous DNA sequence that can be successfully packaged into the resuting phage particles is reduced. M13, T7 and Lambda are examples of suitable phages for this method.

A variation on (Method B) the involves adding a DNA sequence encoding a nucleotide binding domain to the DNA in the phage genome encoding the heterologous peptide be displayed, and further adding the corresponding nucleotide binding site to the phage genome. This causes the heterologous peptide to become directly attached to the phage genome. This peptide/genome complex is then packaged into a phage particle which displays the heterologous peptide. This method is fully described in WO 99/11785.

The phage particles can then be recovered and used to study the binding characteristics of the heterologous peptide or polypeptide. Once isolated, phagemid or phage DNA can be recovered from the peptide- or polypeptide-displaying phage particle, and this DNA can be replicated via PCR. The PCR product can be used to sequence the heterologous peptide or polypeptide displayed by a given phage particle.

The phage display of single-chain antibodies and fragments thereof, has become a routine means of studying the binding characteristics of these polypeptides. There are numerous books available that review phage display techniques and the biology of the bacteriophage. (See, for example, Phage Display – A Laboratory Manual, Barbas *et al.*, (2001) Cold Spring Harbour Laboratory Press).

A third phage display method (Method C) relies on the fact that heterologous polypeptides having a cysteine residue at a desired location can be expressed in a

soluble form by a phagemid or phage genome, and caused to associate with a modified phage surface protein also having a cysteine residue at a surface exposed position, via the formation of a disulphide linkage between the two cysteines. WO 01/ 05950 details the use of this alternative linkage method for the expression of single-chain antibody-derived peptides.

Brief Description of the Invention

Native TCR's are heterodimers which have lengthy transmembrane domains which are essential to maintain their stability as functional dimers. As discussed above, TCRs are useful for research and therapeutic purposes in their soluble forms so display of the insoluble native form has little utility. On the other hand, soluble stable forms of TCRs have proved difficult to design, and since most display methods appear to have been described only for monomeric peptides and polypeptides, display methods suitable for soluble dimeric TCRs have not been investigated. Furthermore, since the functionality of the displayed TCR depends on proper association of the variable domains of the TCR dimer, successful display of a functional dimeric TCR is not trivial.

WO 99/18129 contains the statement: "DNA constructs encoding the sc-TCR fusion proteins can be used to make a bacteriophage display library in accordance with methods described in pending U.S. application Serial No. 08/813.781 filed on March 7, 1997, the disclosure of which is incorporated herein by reference.", but no actual description of such display is included in this application. However, The inventors of this application published a paper (Weidanz (1998) J Immunol Methods **221** 59-76) that demonstrates the display of two murine scTCRs on phage particles.

WO 01/62908 discloses methods for the phage display of scTCRs and scTCR/ Ig fusion proteins. However, the functionality (specific pMHC binding) of the constructs disclosed was not assessed.

Finally, a retrovirus-mediated method for the display of diverse TCR libraries on the surface of immature T cells has been demonstrated for a murine TCR. The library of

mutated TCRs displayed of the surface of the immature T cells was screened by flow cytometry using pMHC tetramers, and this lead to the identification TCR variants that were either specific for the cognate pMHC, or a variant thereof. (Helmut *et al.*, (2000) PNAS **97** (26) 14578-14583)

This invention is based in part on the finding that single chain and dimeric TCRs can be expressed as surface fusions to proteinaceous particles, and makes available proteinaceous particles displaying alpha/beta-analogue and gamma/delta-analogue scTCR and dTCR constructs. The proteinaceous particles on which the TCRs are displayed include self-aggregating particle-forming proteins, phage, virus-derived, ribosome particles and cells with a cell surface protein or polypeptide molecules to which the TCR is covalently linked. Such proteinaceous particle-displayed TCRs are useful for purification and screening purposes, particularly as a diverse library of particle displayed TCRs for biopanning to identify TCRs with desirable characteristics such as high affinity for the target MHC-peptide complex. In the latter connection, particle-displayed scTCRs may be useful for identification of the desired TCR, but that information may be better applied to the construction of analogous dimeric TCRs for ultimate use in therapy. The invention also includes high affinity TCRs identifiable by these methods

Detailed Description of the Invention

In one broad aspect, he present invention provides a proteinaceous particle displaying on its surface a T-cell receptor (TCR), characterised in that

(i) the proteinaceous particle is a ribosome and the TCR is a single chain TCR (scTCR) polypeptide, or dimeric TCR (dTCR) polypeptide pair, or

(ii) the proteinaceous particle is a phage particle, or a cell with cell surface protein or polypeptide molecules to which the TCR is covalently linked, and the TCR is a human scTCR or a human dTCR polypeptide pair, or

(iii) the proteinaceous particle is a phage particle, or a cell with cell surface protein or polypeptide molecules to which the TCR is covalently linked, and the TCR is a non-human dTCR polypeptide pair, or

(iv) the proteinaceous particle is a phage particle, or a cell with cell surface protein or polypeptide molecules to which the TCR is covalently linked, and the TCR is a scTCR polypeptide comprising TCR amino acid sequences corresponding to extracellular constant and variable domain sequences present in native TCR chains and a linker sequence, the latter linking a variable domain sequence corresponding to that of one chain of a native TCR to a constant domain sequence corresponding to a constant domain sequence of another native TCR chain, and a disulfide bond which has no equivalent in native T cell receptors links residues of the constant domain sequences.

In one preferred embodiment, the invention provides A proteinaceous particle, displaying on its surface a dimeric T-cell receptor (dTCR) polypeptide pair, or a single chain T-cell receptor (scTCR) polypeptide wherein the dTCR polypeptide pair is constituted by TCR amino acid sequences corresponding to extracellular constant and variable domain sequences present in native TCR chains, and the scTCR is constituted by TCR amino acid sequences corresponding to extracellular constant and variable domain sequences present in native TCR chains and a linker sequence, the latter linking a variable domain sequence corresponding to that of one chain of a native TCR to a constant domain sequence corresponding to a constant domain sequence of another native TCR chain; wherein the variable domain sequences of the dTCR polypeptide pair or scTCR polypeptide are mutually orientated substantially as in native TCRs, and in the case of the scTCR polypeptide a disulfide bond which has no equivalent in native T cell receptors links residues of the polypeptide.

In the case of αβ scTCRs or dTCRs displayed according to the invention, a requirement that the variable domain sequences of the α and β segments are mutually orientated substantially as in native αβ T cell receptors is merely an alternative way of saying that the TCRs are functional, and this can be tested by confirming that the

molecule binds to the relevant TCR ligand (pMHC complex, CD1-antigen complex, superantigen or superantigen/pMHC complex) - if it binds, then the requirement is met. Interactions with pMHC complexes can be measured using a Biacore 3000™ or Biacore 2000™ instrument. WO99/6120 provides detailed descriptions of the methods required to analyse TCR binding to MHC-peptide complexes. These methods are equally applicable to the study of TCR/ CD1 and TCR/superantigen interactions. In order to apply these methods to the study of TCR/CD1 interactions soluble forms of CD1 are required, the production of which are described in (Bauer (1997) Eur J Immunol **27** (6) 1366-1373). In the case of γδ TCRs of the present invention the cognate ligands for these molecules are unknown therefore secondary means of verifying their conformation such as recognition by antibodies can be employed. The monoclonal antibody MCA991T (available from Serotec), specific for the δ chain variable region, is an example of an antibody appropriate for this task.

The scTCRs or dTCRs of the present invention may be displayed on proteinaceous particles, for example phage particles, preferably filamentous phage particles, by, for example, the following two means:

(i) The C-terminus of one member of the dTCR polypeptide pair, or the C-terminus of the scTCR polypeptide, or the C-terminus of a short peptide linker attached to the C-terminus of either, can be directly linked by a peptide bond to a surface exposed residue of the proteinaceous particle. For example, the said surface exposed residue is preferably at the N-terminus of the gene product of bacteriophage gene III or gene VIII; and

(ii) The C-terminus of one member of the dTCR polypeptide pair, or the C-terminus of the scTCR polypeptide, or the C-terminus of a short peptide linker attached to the C-terminus of either, is linked by a disulfide bond to a surface exposed cysteine residue of the proteinaceous particle via an introduced cysteine residue. For example, the said surface exposed residue is again preferably at the N-terminus of the gene product of bacteriophage gene III or gene VIII.

Method (i) above is preferred. In the case of a scTCR, nucleic acid encoding the TCR may be fused to nucleic acid encoding the particle forming protein or a surface protein of the replicable particle such as a phage or cell. Alternatively, nucleic acid representing mRNA but without a stop codon, or fused to puromycin RNA may be translated by ribosome such that the TCR remains fused to the ribosome particle. In the case of a dTCR, nucleic acid encoding one chain of the TCR may be fused to nucleic acid encoding the particle forming protein or a cell surface protein of the replicable particle such as a phage or cell, and the second chain of the TCR polypeptide pair may be allowed to associate with the resultant expressed particle displaying the first chain. Proper functional association of the two chains may be assisted by the presence of cysteines in the constant domain of the two chains which are capable of forming an interchain disulfide bond, as more fully discussed below.

The displayed scTCR

The displayed scTCR polypeptide may be, for example, one which has

a first segment constituted by an amino acid sequence corresponding to a TCR α or δ chain variable domain sequence fused to the N terminus of an amino acid sequence corresponding to a TCR α chain constant domain extracellular sequence,

a second segment constituted by an amino acid sequence corresponding to a TCR β or γ chain variable domain fused to the N terminus of an amino acid sequence corresponding to TCR β chain constant domain extracellular sequence,

a linker sequence linking the C terminus of the first segment to the N terminus of the second segment, or vice versa, and

a disulfide bond between the first and second chains, said disulfide bond being one which has no equivalent in native αβ or γδ T cell receptors,

the length of the linker sequence and the position of the disulfide bond being such that the variable domain sequences of the first and second segments are mutually orientated substantially as in native αβ or γδ T cell receptors.

Alternatively, the displayed scTCR may be one which has

a first segment constituted by an amino acid sequence corresponding to a TCR α or δ chain variable domain

a second segment constituted by an amino acid sequence corresponding to a TCR β or γ chain variable domain sequence fused to the N terminus of an amino acid sequence corresponding to a TCR β chain constant domain extracellular sequence, and

a linker sequence linking the C terminus of the first segment to the N terminus of the second segment,

PROVIDED THAT where the proteinaceous particle is a phage, the scTCR corresponds to a human TCR; or

One which has

a first segment constituted by an amino acid sequence corresponding to a TCR β or γ chain variable domain

a second segment constituted by an amino acid sequence corresponding to a TCR α or δ chain variable domain sequence fused to the N terminus of an amino acid sequence corresponding to a TCR α chain constant domain extracellular sequence, and

a linker sequence linking the C terminus of the first segment to the N terminus of the second segment

PROVIDED THAT where the proteinaceous particle is a phage, the scTCR corresponds to a human TCR.

The displayed dTCR

The dTCR which is displayed on the proteinaceous particle may be one which is constituted by

a first polypeptide wherein a sequence corresponding to a TCR α or δ chain variable region sequence is fused to the N terminus of a sequence corresponding to a TCR α chain constant domain extracellular sequence, and

a second polypeptide wherein a sequence corresponding to a TCR β or γ chain variable domain sequence fused to the N terminus a sequence corresponding to a TCR β chain constant domain extracellular sequence,

the first and second polypeptides being linked by a disulfide bond which has no equivalent in native αβ or γδ T cell receptors.

Preferably, the dTCR is displayed on a filamentous phage particle and is one which is constituted by

a first polypeptide wherein a sequence corresponding to a TCR α chain variable domain sequence is fused to the N terminus of a sequence corresponding to a TCR α chain constant domain extracellular sequence, and

a second polypeptide wherein a sequence corresponding to a TCR β chain variable domain sequence is fused to the N terminus a sequence corresponding to a TCR β chain constant domain extracellular sequence,

the first and second polypeptides being linked by a disulfide bond between cysteine residues substituted for Thr 48 of exon 1 of TRAC*01 and Ser 57 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof,

the C-terminus of one member of the dTCR polypeptide pair being linked by a peptide bond to a coat protein of the phage.

dTCR Polypeptide Pair and scTCR Polypeptide

The constant domain extracellular sequences present in the scTCRs or dTCRs preferably correspond to those of a human TCR, as do the variable domain sequences. However, the correspondence between such sequences need not be 1:1 on an amino acid level. N- or C-truncation, and/or amino acid deletion and/or substitution relative to the corresponding human TCR sequences is acceptable. In particular, because the constant domain extracellular sequences present in the first and second segments are not directly involved in contacts with the ligand to which the scTCR or dTCR binds, they may be shorter than, or may contain substitutions or deletions relative to, extracellular constant domain sequences of native TCRs.

The constant domain extracellular sequence present in one of the dTCR polypeptide pair, or in the first segment of a scTCR polypeptide may include a sequence corresponding to the extracellular constant Ig domain of a TCR α chain, and/or the constant domain extracellular sequence present in the other member of the pair or second segment may include a sequence corresponding to the extracellular constant Ig domain of a TCR β chain.

In one embodiment of the invention, one member of the dTCR polypeptide pair, or the first segment of the scTCR polypeptide, corresponds to substantially all the variable

domain of a TCR α chain fused to the N terminus of substantially all the extracellular domain of the constant domain of an TCR α chain; and/or the other member of the pair or second segment corresponds to substantially all the variable domain of a TCR β chain fused to the N terminus of substantially all the extracellular domain of the constant domain of a TCR β chain.

In another embodiment, the constant domain extracellular sequences present in the dTCR polypeptide pair, or first and second segments of the scTCR polypeptide, correspond to the constant domains of the α and β chains of a native TCR truncated at their C termini such that the cysteine residues which form the native inter-chain disulfide bond of the TCR are excluded. Alternatively those cysteine residues may be substituted by another amino acid residue such as serine or alanine, so that the native disulfide bond is deleted. In addition, the native TCR β chain contains an unpaired cysteine residue and that residue may be deleted from, or replaced by a non-cysteine residue in, the β sequence of the scTCR of the invention.

In one particular embodiment of the invention, the TCR α and β chain variable domain sequences present in the dTCR polypeptide pair, or first and second segments of the scTCR polypeptide, may together correspond to the functional variable domain of a first TCR, and the TCR α and β chain constant domain extracellular sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a second TCR, the first and second TCRs being from the same species. Thus, the α and β chain variable domain sequences present in dTCR polypeptide pair, or first and second segments of the scTCR polypeptide, may correspond to those of a first human TCR, and the α and β chain constant domain extracellular sequences may correspond to those of a second human TCR. For example, A6 Tax sTCR constant domain extracellular sequences can be used as a framework onto which heterologous α and β variable domains can be fused.

In another embodiment of the invention, the TCR δ and γ chain variable domain sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide respectively, may together correspond to the functional variable domain of a first TCR, and the TCR α and β chain constant domain extracellular sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide respectively, may correspond to those of a second TCR, the first and second TCRs being from the same species. Thus the δ and γ chain variable domain sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a first human TCR, and the α and β chain constant domain extracellular sequences may correspond to those of a second human TCR. For example, A6 Tax sTCR constant domain extracellular sequences can be used as a framework onto which heterologous γ and δ variable domains can be fused.

In one particular embodiment of the invention, the TCR α and β, or δ and γ chain variable domain sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may together correspond to the functional variable domain of a first human TCR, and the TCR α and β chain constant domain extracellular sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a second non-human TCR, Thus the α and β, or δ and γ chain variable domain sequences present dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a first human TCR, and the α and β chain constant domain extracellular sequences may correspond to those of a second non-human TCR. For example, murine TCR constant domain extracellular sequences can be used as a framework onto which heterologous human α and β TCR variable domains can be fused.

Linker in the scTCR Polypeptide

For scTCR-displaying proteinaceous particles of the present invention, a linker sequence links the first and second TCR segments, to form a single polypeptide strand. The linker sequence may, for example, have the formula -P-AA-P- wherein P is proline and AA represents an amino acid sequence wherein the amino acids are glycine and serine.

For the scTCR displayed by proteinaceous particles of the present invention to bind to a ligand, MHC-peptide complex in the case of αβ TCRs, the first and second segments are paired so that the variable domain sequences thereof are orientated for such binding. Hence the linker should have sufficient length to span the distance between the C terminus of the first segment and the N terminus of the second segment, or vice versa. On the other hand excessive linker length should preferably be avoided, in case the end of the linker at the N-terminal variable domain sequence blocks or reduces bonding of the scTCR to the target ligand.

For example, in the case where the constant domain extracellular sequences present in the first and second segments correspond to the constant domains of the α and β chains of a native TCR truncated at their C termini such that the cysteine residues which form the native interchain disulfide bond of the TCR are excluded, and the linker sequence links the C terminus of the first segment to the N terminus of the second segment.

The linker sequence may consist of, for example, from 26 to 41 amino acids, preferably 29, 30, 31 or 32 amino acids, or 33, 34, 35 or 36 amino acids. Particular linkers have the formula -PGGG-$(SGGGG)_5$-P- and -PGGG-$(SGGGG)_6$-P- wherein P is proline, G is glycine and S is serine.

Inter-chain Disulfide bond

A principle characterising feature of the preferred dTCRs and scTCRs displayed by proteinaceous particles of the present invention, is a disulfide bond between the constant domain extracellular sequences of the dTCR polypeptide pair or first and second segments of the scTCR polypeptide. That bond may correspond to the native inter-chain disulfide bond present in native dimeric αβ TCRs, or may have no counterpart in native TCRs, being between cysteines specifically incorporated into the constant domain extracellular sequences of dTCR polypeptide pair or first and second segments of the scTCR polypeptide. In some cases, both a native and a non-native disulfide bond may be desirable.

The position of the disulfide bond is subject to the requirement that the variable domain sequences of dTCR polypeptide pair or first and second segments of the scTCR polypeptide are mutually orientated substantially as in native αβ or γδ T cell receptors.

The disulfide bond may be formed by mutating non-cysteine residues on the first and second segments to cysteine, and causing the bond to be formed between the mutated residues. Residues whose respective β carbons are approximately 6 Å (0.6 nm) or less, and preferably in the range 3.5 Å (0.35 nm) to 5.9 Å (0.59 nm) apart in the native TCR are preferred, such that a disulfide bond can be formed between cysteine residues introduced in place of the native residues. It is preferred if the disulfide bond is between residues in the constant immunoglobulin domain, although it could be between residues of the membrane proximal domain. Preferred sites where cysteines can be introduced to form the disulfide bond are the following residues in exon 1 of TRAC*01 for the TCR α chain and TRBC1*01 or TRBC2*01 for the TCR β chain:

TCR α chain	TCR β chain	Native β carbon separation (nm)
Thr 48	Ser 57	0.473
Thr 45	Ser 77	0.533
Tyr 10	Ser 17	0.359
Thr 45	Asp 59	0.560
Ser 15	Glu 15	0.59

The following motifs in the respective human TCR chains may be used to identify the residue to be mutated (the shaded residue is the residue for mutation to a cysteine).

α Chain Thr 48: DSDVYITDKTVLDMRSMDFK (amino acids 39-58 of exon 1 of the TRAC*01 gene) **(SEQ ID 1)**

α Chain Thr 45: QSKDSDVYITDKTVLDMRSM(amino acids 36-55 of exon 1 of the TRAC*01 gene) **(SEQ ID 2)**

α Chain Tyr 10: DIQNPDPAVYQLRDSKSSDK(amino acids 1-20 of exon 1 of the TRAC*01 gene) **(SEQ ID 3)**

α Chain Ser 15: DPAVYQLRDSKSSDKSVCLF(amino acids 6-25 of exon 1 of the TRAC*01 gene) **(SEQ ID 4)**

β Chain Ser 57: NGKEVHSGVSTDPQPLKEQP(amino acids 48- 67 of exon 1 of the TRBC1*01 & TRBC2*01 genes) **(SEQ ID 5)**

β Chain Ser 77: ALNDSRYALSSRLRVSATFW(amino acids 68- 87 of exon 1 of the TRBC1*01 & TRBC2*01 genes) **(SEQ ID 6)**

β Chain Ser 17: PPEVAVFEPSEAEISHTQKA(amino acids 8- 27 of exon 1 of the TRBC1*01 & TRBC2*01 genes) **(SEQ ID 7)**

β Chain Asp 59: KEVHSGVSTDPQPLKEQPAL(amino acids 50- 69 of exon 1 of the TRBC1*01 & TRBC2*01 genes gene) **(SEQ ID 8)**

β Chain Glu 15: VFPPEVAVFEPSEAEISHTQ(amino acids 6- 25 of exon 1 of the TRBC1*01 & TRBC2*01 genes) **(SEQ ID 9)**

In other species, the TCR chains may not have a region which has 100% identity to the above motifs. However, those of skill in the art will be able to use the above motifs to identify the equivalent part of the TCR α or β chain and hence the residue to be mutated to cysteine. Alignment techniques may be used in this respect. For example, ClustalW, available on the European Bioinformatics Institute website (http://www.ebi.ac.uk/index.html) can be used to compare the motifs above to a particular TCR chain sequence in order to locate the relevant part of the TCR sequence for mutation.

The present invention includes within its scope proteinaceous particle-displayed αβ and γδ-analogue scTCRs, as well as those of other mammals, including, but not limited to, mouse, rat, pig, goat and sheep. As mentioned above, those of skill in the art will be able to determine sites equivalent to the above-described human sites at which cysteine residues can be introduced to form an inter-chain disulfide bond. For example, the following shows the amino acid sequences of the mouse Cα and Cβ soluble domains, together with motifs showing the murine residues equivalent to the human residues mentioned above that can be mutated to cysteines to form a TCR interchain disulfide bond (where the relevant residues are shaded):

Mouse Cα soluble domain:
PYIQNPEPAVYQLKDPRSQDSTLCLFTDFDSQINVPKTMESGTFITDKTVLDMK
AMDSKSNGAIAWSNQTSFTCQDIFKETNATYPSSDVP **(SEQ ID 10)**

Mouse Cβ soluble domain:

EDLRNVTPPKVSLFEPSKAEIANKQKATLVCLARGFFPDHVELSWWVNGREV
HSGVSTDPQAYKESNYSYCLSSRLRVSATFWHNPRNHFRCQVQFHGLSEEDK
WPEGSPKPVTQNISAEAWGRAD **(SEQ ID 11)**

Murine equivalent of human α Chain Thr 48: ESGTFITDKTVLDMKAMDSK **(SEQ ID 12)**

Murine equivalent of human α Chain Thr 45: KTMESGTFITDKTVLDMKAM **(SEQ ID 13)**

Murine equivalent of human α Chain Tyr 10: YIQNPEPAVYQLKDPRSQDS **(SEQ ID 14)**

Murine equivalent of human α Chain Ser 15: AVYQLKDPRSQDSTLCLFTD **(SEQ ID 15)**

Murine equivalent of human β Chain Ser 57: NGREVHSGVSTDPQAYKESN **(SEQ ID 16)**

Murine equivalent of human β Chain Ser 77: KESNYSYCLSSRLRVSATFW **(SEQ ID 17)**

Murine equivalent of human β Chain Ser 17: PPKVSLFEPSKAEIANKQKA **(SEQ ID 18)**

Murine equivalent of human β Chain Asp 59: REVHSGVSTDPQAYKESNYS **(SEQ ID 19)**

Murine equivalent of human β Chain Glu 15: VTPPKVSLFEPSKAEIANKQ **(SEQ ID 20)**

As discussed above, the A6 Tax sTCR extracellular constant domains can be used as framework onto which heterologous variable domains can be fused. It is preferred that the heterologous variable domain sequences are linked to the constant domain sequences at any point between the disulfide bond and the N termini of the constant domain sequences. In the case of the A6 Tax TCR α and β constant domain sequences, the disulfide bond may be formed between cysteine residues introduced at amino acid residues 158 and 172 respectively. Therefore it is preferred if the

heterologous α and β chain variable domain sequence attachment points are between residues 159 or 173 and the N terminus of the α or β constant domain sequences respectively.

TCR Display.

The preferred *in-vivo* TCR display method for biopanning to identify TCRs having desirable properties such as high affinity for a target peptide-MHC complex is phage display.

Firstly, a DNA library is constructed that encodes a diverse array of mutated scTCRs or dTCRs. This library is constructed by using DNA encoding a native TCR as the template for amplification. There are a number of suitable methods, known to those skilled in the art, for the introduction of the desired mutations into the TCR DNA, and hence the finally expressed TCR protein. For example error-prone PCR (EP-TCR), DNA shuffling techniques, and the use of bacterial mutator strains such as XL-1-Red are convenient means of introducing mutations into the TCR sequences. It is particularly preferred if these mutations are introduced into defined domain of the TCRs. For example, mutations in the variable domain, particularly the complementarity-determining regions (CDRs) and/or framework regions are likely to be the most appropriate sites for the introduction of mutations leading to the production of a diverse library of TCRs for the production of TCRs with enhanced ligand-binding properties. EP-PCR is an example of a method by which such 'region-specific' mutations can be introduced into the TCRs. EP-PCR primers are used that are complementary to DNA sequences bordering the region to be mutated to amplify multiple copies of this region of the TCR DNA that contain a controllable level of random mutations. These DNA sequences encoding mutated regions are inserted into the DNA sequences, which encode the non-mutagenised sections of the TCR, by ligation or overlapping PCR. The DNA encoding the TCR with mutated region can then be ligated onto DNA encoding a heterologous polypeptide in order to produce a fusion protein suitable for display. In the case of phage-display the expression vector utilised is either a phagemid or a phage gemone vector in which the TCR DNA can be

ligated to DNA encoding a surface protein, preferably the gIII or gVIII surface protein. In the case of a scTCR such ligation is performed as for phage display of any monomeric peptide or polypeptide. In the case of dTCRs, only one of the TCR chains is ligated as aforesaid. The other chain is encoded in nucleic acid for co-expression with phagemid and helper phage nucleic acid, so that the expressed second chain finds and associates with the expressed phage with surface displayed first chain. In both cases, as discussed in more detail above, properly positioned cysteines in the constant domains are helpful in causing the variable domains of the TCR to adopt their functional positions, through the formation of a disulfide bond by those cysteines.

For expression, an expression vector comprising (a) nucleic acid encoding one chain of a dTCR polypeptide pair, and (b) the other chain of a dTCR polypeptide pair fused to a nucleic acid sequence encoding a particle forming protein, or a cell surface protein; or nucleic acid encoding a scTCR polypeptide fused to a nucleic acid sequence encoding a particle forming protein or a cell surface protein, the dTCR pair, or a composition comprising a first vector comprising nucleic acid (a) and a second vector comprising nucleic acid (b), are contacted with host cells capable of causing the expression of the encoded genetic material under conditions suitable to allow the transformation of said cells. Such expression vectors, expression systems comprising phagemid or phage genome vectors encoding dTCRs and scTCRs, and host cells harbouring them form additional aspects of the current invention. In a preferred embodiment of the invention the phagemid or phage genome vectors are derived from filamentous phage.

The transformed cells are then incubated to allow the expression of the TCR-displaying proteinaceous particles. These particles can then be used for screening or in assays to identify TCR variants with specific enhanced characteristics. Any particles that possess the enhanced characteristics under investigation can then be isolated. The DNA encoding these TCRs can then be amplified by PCR and the sequence determined.

It is known that high expression levels of an exogenous polypeptide may be toxic to the host cell. In such cases, either a host strain which is more tolerant of the exogenous polypeptide must be found, or the expression levels in the host cell must be limited to a level which is tolerated. For example (Beekwilder *et al.*, (1999) Gene *228* (1-2) 23-31) report that only mutated forms of a potato protease inhibitor (PI2) which contained deletions or amber stop codons would be successfully selected from a phage display library. In the present case, an observation in the course of the work reported in the Examples herein suggests that it may be desirable to limit the expression levels of protein particle-displayed TCRs of the invention, at least in some strains of *E. coli.* Thus, the A6 TCR selected in Example 4 after repeated rounds of culture was shown to be derived from cells in which the phagemid had mutated relative to that introduced at the start. The mutation had created an 'opal' stop codon in the TCR β chain. This codon is 'read-through' with low frequency by ribosomes of the *E.coli* strain utilised resulting in the insertion of a tryptophan residue at this site and a much reduced overall level of full-length β chain expression.

There are several strategies for limiting the expression levels of an exogenous polypeptide from a given expression ~~vector~~ system in a host which may be suitable for the limiting the expression levels of a scTCR, or one, or both TCR chains of a dTCR . For example:

Use of a weak promoter sequence – The level of expression obtained for a given gene product, such as the TCR α or β chain, can be tailored by using promoter sequences of varying strengths. The lambda phage P_{RM} promoter is an example of a weak promoter.

Mutated ribosome binding sites (RBS's) – Mutating a single nucleic acid in the RBS associated with a gene product, such as the TCR α or β chain, can result in a reduced level of expression. For example, mutating a wild-type AGGA sequence to AGGG.

Mutated 'start codons' - Mutating a single nucleic acid in the start codon associated with a gene product, such as the TCR α or β chain, can also result in a reduced level of expression. For example, mutating a wild-type AUG start codon to GUG.

Miss-sense suppressor mutations – These are inserted within the TCR β chain coding regions. Examples include the 'opal' stop codon (UGA), this 'leaky' stop codon results in the low frequency insertion of a tryptophan amino acid and read-through of the rest of the coding sequence.

Metabolite-mediated modification of promoter strength – The level of expression of a gene product, such as the TCR α or β chain, under the control of certain promoters can be down-regulated by the addition of a relevant metabolite to the cells containing the promoter. For example, glucose additions can be used to down-regulate expression of a gene product under the control of a Lac promoter.

Codon usage - Bacterial cells and, for example, mammalian cells have different 'preferences' relating to the codons they use to encode certain amino acids. For example, bacterial cells most commonly use the CGU codon to encode arginine whereas eucaryotic cells most commonly use AGA. It is possible to reduce the level of expression of a gene product, such as the TCR α or β chain, by utilising DNA sequences that contain a number of codons less preferred by the expression system being utilised.

Details relating to the above means of down-regulating gene product expression can be found in (Glass (1982) Gene Function – *E.coli* and its heritable elements, Croom Helm) and (Rezinoff (1980) The Operon 2nd Edition, Cold Spring Harbor Laboratory).

It is also known that supplying bacterial cultures with a relatively high concentration of a sugar such as sucrose can increase periplasmic expression levels of soluble proteins. (See for example (Sawyer *et al.*, (1994) Protein Engineering 7 **(11)** 1401-1406))

After expression, correct pairing of the scTCR polypeptide variable domain sequences is preferably assisted by the introduction of a disulfide bond in the extracellular constant domain of the scTCR. Without wanting to be limited by theory, the novel disulfide bond is believed to provide extra stability to the scTCR during the folding process and thereby facilitating correct pairing of the first and second segments.

Also as mentioned above, for dTCR phage display, one of the dTCR polypeptide pair is expressed as if it were eventually to be displayed as a monomeric polypeptide on the phage, and the other of the dTCR polypeptide pair is co-expressed in the same host cell. As the phage particle self assembles, the two polypeptides self associate for display as a dimer on the phage. Again, in the preferred embodiment of this aspect of the invention, correct folding during association of the polypeptide pair is assisted by a disulfide bond between the constant sequences, as discussed above. Further details of a procedure for phage display of a dTCR having an interchain disulfide bond appear in the Examples herein.

As an alternative, the phage displaying the first chain of the dTCR may be expressed first, and the second chain polypeptide may be contacted with the expressed phage in a subsequent step, for association as a functional dTCR on the phage surface.

The preferred *in-vitro* TCR display method for biopanning to identify TCRs having desirable properties such as high affinity for a target peptide-MHC complex is ribosomal display. Firstly, a DNA library is constructed that encodes a diverse array of mutated scTCRs or dTCR polypeptides using the techniques discussed above. The DNA library is then contacted with RNA polymerase in order to produce a complementary mRNA library. Optionally, for mRNA display techniques the mRNA sequences can then be ligated to a DNA sequence comprising a puromycin binding site. These genetic constructs are then contacted with ribosomes in-vitro under conditions allowing the translation of the scTCR polypeptide or the first polypeptide of the dTCR pair. In the case of the dTCR, the second of the polypeptide pairs is separately expressed and contacted with the ribosome-displayed first polypeptide, for

association between the two, preferably assisted by the formation of the disulphide bond between constant domains. Alternatively, mRNA encoding both chains of the TCR may be contacted with ribosomes in-vitro under conditions allowing the translation of the TCR chains such that a ribosome displaying a dTCR is formed. These scTCR- or dTCR-displaying ribosomes can then used for screening or in assays to identify TCR variants with specific enhanced characteristics. Any particles that possess the enhanced characteristics under investigation can then be isolated. The mRNA encoding these TCRs can then converted to the complementary DNA sequences using reverse transcriptase. This DNA can then be amplified by PCR and the sequence determined.

Additional Aspects

A proteinaceous particle displaying a scTCR or dTCR (which preferably is constituted by constant and variable sequences corresponding to human sequences) of the present invention may be provided in substantially pure form, or as a purified or isolated preparation. For example, it may be provided in a form which is substantially free of other proteins.

A phage particle displaying a plurality of scTCRs or dTCRs of the present invention may be provided in a multivalent complex. Thus, the present invention provides, in one aspect, a multivalent T cell receptor (TCR) complex, which comprises a phage particle displaying a plurality of scTCRs or dTCRs as described herein. Each of the plurality of said scTCRs or dTCRs is preferably identical.

In a further aspect, the invention provides a method for detecting TCR ligand complexes, which comprises:

a. providing a TCR-displaying proteineaceous particle of the current invention
b. contacting the TCR-displaying phage with the putative ligand complexes; and

detecting binding of the TCR-displaying proteinaceous particle to the putative ligand complexes.

TCR ligands suitable for identification by the above method include, but are not limited to, peptide-MHC complexes.

Isolation of TCR variants with enhanced characteristics

A further aspect of the invention is a method for the identification of TCRs with a specific characteristic, said method comprising subjecting a diverse library of TCRs displayed on proteinaceous particles to a selection process which selects for said characteristic, and isolating proteinaceous particles which display a TCR having said characteristic, and optionally to an amplification process to multiply the isolated particles, and/or a screening process which measures said characteristic, identifying those proteinaceous particles which display a TCR with the desired characteristic and isolating these proteinaceous particles, and optionally to an amplification process to multiply the isolated particles.

The DNA sequences encoding the variant TCRs can then be obtained and amplified by PCR to allow the sequences to be determined. The characteristics that can be enhanced include, but are not limited to, ligand binding affinity and construct stability.

Screening Use

The TCR-displaying proteinaceous particles of the present invention are capable of utilisation in screening methods designed to identify modulators, including inhibitors, of the TCR-mediated cellular immune synapse.

As is know to those skilled in the art there are a number of assay formats that provide a suitable basis for protein-protein interaction screens of this type.

Amplified Luminescent Proximity Homogeneous Assay systems such as the AlphaScreen™, rely on the use of "Donor" and "Acceptor" beads that are coated with

a layer of hydrogel to which receptor and ligand proteins can be attached. The interaction between these receptor and ligand molecules brings the beads into proximity. When these beads are subject to laser light a photosensitizer in the "Donor" bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with a chemiluminescer in the "Acceptor" bead that further activates fluorophores contained within the same bead. The fluorophores subsequently emit light at 520-620 nm, this signals that the receptor-ligand interaction has occurred. The presence of an inhibitor of the receptor-ligand interaction causes this signal to be diminished.

Surface Plasmon Resonance (SPR) is an interfacial optical assay, in which one binding partner (normally the receptor) is immobilised on a 'chip' (the sensor surface) and the binding of the other binding partner (normally the ligand), which is soluble and is caused to flow over the chip, is detected. The binding of the ligand results in an increase in concentration of protein near to the chip surface which causes a change in the refractive index in that region. The surface of the chip is comprised such that the change in refractive index may be detected by surface plasmon resonance, an optical phenomenon whereby light at a certain angle of incidence on a thin metal film produces a reflected beam of reduced intensity due to the resonant excitation of waves of oscillating surface charge density (surface plasmons). The resonance is very sensitive to changes in the refractive index on the far side of the metal film, and it is this signal which is used to detect binding between the immobilised and soluble proteins. Systems which allow convenient use of SPR detection of molecular interactions, and data analysis, are commercially available. Examples are the Iasys™ machines (Fisons) and the Biacore™ machines.

Other interfacial optical assays include total internal reflectance fluorescence (TIRF), resonant mirror (RM) and optical grating coupler sensor (GCS), and are discussed in more detail in Woodbury and Venton (*J. Chromatog. B.* **725** 113-137 (1999)).

The scintillation proximity assay (SPA) has been used to screen compound libraries for inhibitors of the low affinity interaction between CD28 and B7 (K_d probably in the

region of 4 μM (Van der Merwe *et al* J. Exp. Med. **185**:393-403 (1997), Jenh *et al,* Anal Biochem **165**(2) 287-93 (1998)). SPA is a radioactive assay making use of beta particle emission from certain radioactive isotopes which transfers energy to a scintillant immobilised on the indicator surface. The short range of the beta particles in solution ensures that scintillation only occurs when the beta particles are emitted in close proximity to the scintillant. When applied for the detection of protein-protein interactions, one interaction partner is labelled with the radioisotope, while the other is either bound to beads containing scintillant or coated on a surface together with scintillant. If the assay can be set up optimally, the radioisotope will be brought close enough to the scintillant for photon emission to be activated only when binding between the two proteins occurs.

A further aspect of the invention is a method of identifying an inhibitor of the interaction between a TCR-displaying proteinaceous particle of the invention, and a TCR-binding ligand comprising contacting the TCR-displaying proteinaceous particle with a TCR-binding ligand, in the presence of and in the absence of a test compound, and determining whether the presence of the test compound reduces binding of the TCR-displaying proteinaceous particle to the TCR-binding ligand, such reduction being taken as identifying an inhibitor.

A further aspect of the invention is a method of identifying a potential inhibitor of the interaction between an TCR-displaying proteinaceous particle of the invention, and a TCR-binding ligand, for example a MHC-peptide complex, comprising contacting the TCR-displaying proteinaceous particle or TCR-binding ligand partner with a test compound and determining whether the test compound binds to the TCR-displaying proteinaceous particle and/or the TCR-binding ligand, such binding being taken as identifying a potential inhibitor. This aspect of the invention may find particular utility in interfacial optical assays such as those carried out using the Biacore™ system.

High Affinity TCRs

The present invention also makes available mutated TCRs specific for a given TCR ligand with higher affinity for said TCR ligand than the wild-type TCR. These high affinity TCRs are expected to be particularly useful for the diagnosis and treatment of disease.

As used herein the term 'high affinity TCR' refers to a mutated scTCR or dTCR which interacts with a specific TCR ligand and either:has a Kd for the said TCR ligand less than that of a corresponding native TCR as measured by Surface Plasmon Resonance, or has an off-rate (k_{off}) for the said TCR ligand less than that of a corresponding native TCR as measured by Surface Plasmon Resonance.

High affinity scTCRs or dTCRs of the present invention are preferably mutated relative to the native TCR in at least one complementarity determining region and/or framework regions.

In one aspect of the present invention the TCR ligand for which a given high affinity TCR is specific is a peptide-MHC complex (pMHC).

In another aspect of the present invention the TCR ligand for which a given high affinity TCR is specific is an MHC type or types.

In a further aspect of the present invention the TCR ligand for which a given high affinity TCR is specific is the HLA-A2 tax peptide (LLFGYPVYV) **(SEQ ID 21)** complex.

In a further aspect of the present invention the TCR ligand for which a given high affinity TCR is specific is the HLA-A2 NY-ESO peptide (SLLMITQC) **(SEQ ID 22)** complex.

A high affinity scTCR or one or both of the high affinity dTCR chains may be labelled with an imaging compound, for example a label that is suitable for diagnostic purposes. Such labelled high affinity TCRs are useful in a method for detecting a TCR ligand selected from CD1-antigen complexes, bacterial superantigens, and MHC-peptide/superantigen complexes which method comprises contacting the TCR ligand with a high affinity TCR (or a multimeric high affinity TCR complex) which is specific for the TCR ligand; and detecting binding to the TCR ligand. In tetrameric high affinity TCR complexes (formed, for example) using biotinylated heterodimers) fluorescent streptavidin (commercially available) can be used to provide a detectable label. A fluorescently-labelled tetramer is suitable for use in FACS analysis, for example to detect antigen presenting cells carrying the peptide for which the high affinity TCR is specific.

Another manner in which the soluble high affinity TCRs of the present invention may be detected is by the use of TCR-specific antibodies, in particular monoclonal antibodies. There are many commercially available anti-TCR antibodies, such as αF1 and βF1, which recognise the constant domains of the α and β chains, respectively.

A high affinity TCR (or multivalent complex thereof) of the present invention may alternatively or additionally be associated with (e.g. covalently or otherwise linked to) a therapeutic agent which may be, for example, a toxic moiety for use in cell killing, or an immunostimulating agent such as an interleukin or a cytokine. A multivalent high affinity TCR complex of the present invention may have enhanced binding capability for a TCR ligand compared to a non-multimeric wild-type or high affinity T cell receptor heterodimer. Thus, the multivalent high affinity TCR complexes according to the invention are particularly useful for tracking or targeting cells presenting particular antigens *in vitro* or *in vivo*, and are also useful as intermediates for the production of further multivalent high affinity TCR complexes having such uses. The high affinity TCR or multivalent high affinity TCR complex may therefore be provided in a pharmaceutically acceptable formulation for use *in vivo*.

The invention also provides a method for delivering a therapeutic agent to a target cell, which method comprises contacting potential target cells with a high affinity TCR or multivalent high affinity TCR complex in accordance with the invention under conditions to allow attachment of the high affinity TCR or multivalent high affinity TCR complex to the target cell, said high affinity TCR or multivalent high affinity TCR complex being specific for the TCR ligand and having the therapeutic agent associated therewith.

In particular, the soluble high affinity TCR or multivalent high affinity TCR complex can be used to deliver therapeutic agents to the location of cells presenting a particular antigen. This would be useful in many situations and, in particular, against tumours. A therapeutic agent could be delivered such that it would exercise its effect locally but not only on the cell it binds to. Thus, one particular strategy envisages anti-tumour molecules linked to high affinity T cell receptors or multivalent high affinity TCR complexes specific for tumour antigens.

Many therapeutic agents could be employed for this use, for instance radioactive compounds, enzymes (perforin for example) or chemotherapeutic agents (cis-platin for example). To ensure that toxic effects are exercised in the desired location the toxin could be inside a liposome linked to streptavidin so that the compound is released slowly. This will prevent damaging effects during the transport in the body and ensure that the toxin has maximum effect after binding of the TCR to the relevant antigen presenting cells.

Other suitable therapeutic agents include:

- small molecule cytotoxic agents, i.e. compounds with the ability to kill mammalian cells having a molecular weight of less than 700 daltons. Such compounds could also contain toxic metals capable of having a cytotoxic effect. Furthermore, it is to be understood that these small molecule cytotoxic agents also include pro-drugs, i.e. compounds that decay or are converted under physiological conditions to release cytotoxic agents. Examples of such agents include cis-platin,

maytansine derivatives, rachelmycin, calicheamicin, docetaxel, etoposide, gemcitabine, ifosfamide, irinotecan, melphalan, mitoxantrone, sorfimer sodiumphotofrin II, temozolmide, topotecan, trimetreate glucuronate, auristatin E vincristine and doxorubicin;

- peptide cytotoxins, i.e. proteins or fragments thereof with the ability to kill mammalian cells. Examples include ricin, diphtheria toxin, pseudomonas bacterial exotoxin A, DNAase and RNAase;
- radio-nuclides, i.e. unstable isotopes of elements which decay with the concurrent emission of one or more of α or β particles, or γ rays. Examples include iodine 131, rhenium 186, indium 111, yttrium 90, bismuth 210 and 213, actinium 225 and astatine 213; chelating agents may be used to facilitate the association of these radio-nuclides to the high affinity TCRs, or multimers thereof;
- prodrugs, such as antibody directed enzyme pro-drugs;
- immuno-stimulants, i.e. moieties which stimulate immune response. Examples include cytokines such as IL-2, chemokines such as IL-8, platelet factor 4, melanoma growth stimulatory protein, etc, antibodies or fragments thereof, complement activators, xenogeneic protein domains, allogeneic protein domains, viral/bacterial protein domains and viral/bacterial peptides.

Soluble high affinity TCRs or multivalent high affinity TCR complexes of the invention may be linked to an enzyme capable of converting a prodrug to a drug. This allows the prodrug to be converted to the drug only at the site where it is required (i.e. targeted by the sTCR).

A multitude of disease treatments can potentially be enhanced by localising the drug through the specificity of soluble high affinity TCRs. For example, it is expected that the high affinity HLA-A2-tax (LLFGYPVYV) **(SEQ ID 21)** specific A6 TCRs disclosed herein may be used in methods for the diagnosis and treatment of HTLV-1 and that the high affinity HLA-A2-NY-ESO (SLLMITQC) **(SEQ ID 22)** specific NY-ESO TCR disclosed herein may be used in methods for the diagnosis and treatment of cancer.

Viral diseases for which drugs exist, e.g. HIV, SIV, EBV, CMV, would benefit from the drug being released or activated in the near vicinity of infected cells. For cancer, the localisation in the vicinity of tumours or metastasis would enhance the effect of toxins or immunostimulants. In autoimmune diseases, immunosuppressive drugs could be released slowly, having more local effect over a longer time-span while minimally affecting the overall immuno-capacity of the subject. In the prevention of graft rejection, the effect of immunosuppressive drugs could be optimised in the same way. For vaccine delivery, the vaccine antigen could be localised in the vicinity of antigen presenting cells, thus enhancing the efficacy of the antigen. The method can also be applied for imaging purposes.

The soluble high affinity TCRs of the present invention may be used to modulate T cell activation by binding to specific TCR ligand and thereby inhibiting T cell activation. Autoimmune diseases involving T cell-mediated inflammation and/or tissue damage would be amenable to this approach, for example type I diabetes. Knowledge of the specific peptide epitope presented by the relevant pMHC is required for this use.

Therapeutic or imaging high affinity TCRs in accordance with the invention will usually be supplied as part of a sterile, pharmaceutical composition which will normally include a pharmaceutically acceptable carrier. This pharmaceutical composition may be in any suitable form, (depending upon the desired method of administering it to a patient). It may be provided in unit dosage form, will generally be provided in a sealed container and may be provided as part of a kit. Such a kit would normally (although not necessarily) include instructions for use. It may include a plurality of said unit dosage forms.

The pharmaceutical composition may be adapted for administration by any appropriate route, for example parenteral, transdermal or via inhalation, preferably a parenteral (including subcutaneous, intramuscular, or, most preferably intravenous) route. Such compositions may be prepared by any method known in the art of pharmacy, for example by admixing the active ingredient with the carrier(s) or excipient(s) under sterile conditions.

Dosages of the substances of the present invention can vary between wide limits, depending upon the disease or disorder to be treated, the age and condition of the individual to be treated, etc. and a physician will ultimately determine appropriate dosages to be used.

The invention also provides a method for obtaining a high affinity TCR chain, which method comprises incubating such a host cell under conditions causing expression of the high affinity TCR chain and then purifying the polypeptide.

Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis*. The prior art documents mentioned herein are incorporated to the fullest extent permitted by law.

Examples

The invention is further described in the following examples, which do not limit the scope of the invention in any way.

Reference is made in the following to the accompanying drawings in which:

Figures 1a and 1b show respectively the nucleic acid sequences of a soluble A6 TCR α and β chains, mutated so as to introduce a cysteine codon. The shading indicates the introduced cysteine codons.

Figure 2a shows the A6 TCR α chain extracellular amino acid sequence, including the $T_{48} \rightarrow C$ mutation (underlined) used to produce the novel disulphide inter-chain bond, and Figure 2b shows the A6 TCR β chain extracellular amino acid sequence, including the $S_{57} \rightarrow C$ mutation (underlined) used to produce the novel disulphide inter-chain bond.

Figure 3 Outlines the cloning of TCR α and β chains into phagemid vectors. The diagram describes a phage display vector. RSB is the ribosome-binding site. S1 or S2 are signal peptides for secretion of proteins into periplasm of *E. coli*. The * indicates translation stop codon. Either of the TCR α chain or β chain can be fused to phage coat protein, however in this diagram only TCR β chain is fused to phage coat protein.

Figure 4 details the DNA sequence of phagemid pEX746:A6.

Figure 5 expression of phage particle fusions of bacterial coat protein and heterodimeric A6 TCR in *E. coli*. Fusion proteins of heterodimeric A6 TCR::gIII are detected using western blotting. Phage particles are prepared from *E. coli* XL-1-Blue and concentrated with PEG/NaCl. The samples are loaded in reducing or non-reducing sample buffers. Lane 1 is the sample of clone 7 containing correct sequence, and lane 2 is the sample of clone 14 containing a deletion in the α-chain encoding gene. The heterodimeric A6 TCR:gIII fusion protein was detected at 125kDa.

Figure 6 illustrates ELISA detection of pMHC peptide complex binding activity of a heterodimeric A6 TCR displayed on phage. Clone 7 binds specifically to HLA A2-Tax complex. Clone 14 cannot bind to any pMHC, as no TCR is attached to the phage particles.

Figure 7a schematic illustration of the single-chain A6 TCR-C-Kappa DNA ribosome display construct.

Figures 7b and 7c detail the complete DNA coding strand and amino acid sequence of the single-chain A6 TCR-C-Kappa DNA ribosome display construct encoded in pUC19 respectively.

Figure 8 details the DNA sequence of pUC19-T7.

Figure 9 details the DNA sequence of the single-chain A6 TCR-C-Kappa ribosome display construct that was cloned into pUC19-T7.

Figure 10 Western blot showing the detection of in-vitro translated single-chain A6 TCR-C-Kappa using Ambion rabbit reticulocyte lysates.

Figure 11 RT-PCR of the single–chain A6 TCR-C-Kappa mRNA on beads rescued from the ribosome display reactions.

Figure 12a details the DNA sequence of the A6 TCR Clone 9 mutated β chain; the mutated nucleic acid is indicated in bold.

Figure 12b details the amino acid sequence of the A6 TCR Clone 9 mutated β chain, the position corresponding to the introduced opal stop codon is indicated with an *.

Figure 13 details the DNA sequence of the A6 TCR Clone 49 mutated β chain; the mutated nucleic acid is indicated in bold. As this is a 'silent' mutation no change is introduced into the resulting amino acid sequence by this mutation.

Figure 14a details the DNA sequence of the A6 TCR Clone 134 mutated A6 TCR β chain; the mutated nucleic acids are indicated in bold.

Figure 14b details the amino acid sequence of the A6 TCR Clone 134 mutated A6 TCR β chain as tested by BIAcore assay; the mutated amino acids are indicated in bold.

Figure 14c details the amino acid sequence of the A6 TCR Clone 134 mutated A6 TCR β chain as tested by phage ELISA assay; the mutated amino acids are indicated in bold.

Figure 15 BIAcore data for the binding of A6 TCR clone 134 to HLA-A2 Tax and HLA-A2 NY-ESO

Figure 16 BIAcore data used to determine T_{OFF} for the binding of A6 TCR clone 134 to HLA-A2 Tax

Figures 17a and 17b show the DNA sequence of the mutated α and β chains of the NY-ESO TCR respectively

Figures 18a and 18b show the amino acid sequences of the mutated α and β chains of the NY-ESO TCR respectively

Figures 19a and 19b detail the DNA and amino acid sequence of the NY-ESO TCR β chain incorporated into the pEX746:NY-ESO phagemid respectively.

Figure 20 shows the specific binding of phage particles displaying the NY-ESO TCR to HLA-A2-NY-ESO in a phage ELISA assay.

Figure 21 shows the DNA sequence of the DR1α chain incorporating codons encoding the Fos dimerisation peptide attached to the 3' end of the DRA0101 sequence. Shading indicates the Fos codons and the biotinylation tag codons are indicated by in bold text.

Figure 22 shows the DNA sequence of the pAcAB3 bi-cistronic vector used for the expression of Class II HLA-peptide complexes in Sf9 insect cells. The Bgl II restriction site (AGATCT) used to insert the HLA α chain and the BamHI restriction site (GGATCC) used to insert the HLA β chain are indicated by shading.

Figure 23 shows the DNA sequence of the DR1 β chain incorporating codons encoding the Jun dimerisation peptide attached to the 3' end of the DRB0401 sequence and codons encoding an HLA-loaded peptide attached to the 5' end of the DRB0401 sequence. Shading indicates the Jun codons, and the HLA-loaded Flu HA peptide codons are underlined.

Figure 24 shows a BIAcore trace of the binding of the high affinity A6 TCR clone 134 to flowcells coated as follows:

Flow-cell 1 (FC 1) - Blank
Flow-cell 2 (FC 2) - HLA-A2 (LLGRNSFEV) **(SEQ ID 23)**
Flow-cell 3 (FC 3) - HLA-A2 (KLVALGINAV) **(SEQ ID 24)**
Flow-cell 4 (FC 4) - HLA-A2 (LLGDLFGV) **(SEQ ID 25)**

Figure 25 shows a BIAcore trace of the binding of the high affinity A6 TCR clone 134 to flowcells coated as follows:
Flow-cell 1 (FC 1) - Blank
Flow-cell 2 (FC 2) - HLA-B8 (FLRGRAYGL) **(SEQ ID 26)**
Flow-cell 3 (FC 3) - HLA-B27 (HRCQAIRKK) **(SEQ ID 27)**
Flow-cell 4 (FC 4) - HLA- Cw6 (YRSGIIAVV) **(SEQ ID 28)**

Figure 26 shows a BIAcore trace of the binding of the high affinity A6 TCR clone 134 to flowcells coated as follows:
Flow-cell 1 (FC 1) – Blank
Flow-cell 2 (FC 2) – HLA-A24 (VYGFVRACL) **(SEQ ID 29)**
Flow-cell 3 (FC 3) - HLA-A2 (ILAKFLHWL) **(SEQ ID 30)**
Flow-cell 4 (FC 4) - HLA-A2 (LTLGEFLKL) **(SEQ ID 31)**

Figure 27 shows a BIAcore trace of the binding of the high affinity A6 TCR clone 134 to flowcells coated as follows:
Flow-cell 1 (FC 1) – Blank
Flow-cell 2 (FC 2) – HLA-DR1 (PKYVKQNTLKLA) **(SEQ ID 32)**
Flow-cell 3 (FC 3) -HLA-A2 (GILGFVFTL) **(SEQ ID 33)**
Flow-cell 4 (FC 4) - HLA-A2 (SLYNTVATL) **(SEQ ID 34)**

Figure 28 shows a BIAcore trace of the binding of the high affinity A6 TCR clone 134 to flowcells coated as follows:
Flow-cell 1 (FC 1) – Blank
Flow-cell 4 (FC 4) - HLA-A2 (LLFGYPVYV) **(SEQ ID 21)**

Figures 29a and 29b show Biacore plots of the interaction between the soluble high affinity NY-ESO TCR and HLA-A2 NY-ESO.

Figures 30a and 30b show Biacore plots of the interaction between the soluble "wild-type" NY-ESO TCR and HLA-A2 NY-ESO.

Figures 31a and 31b show Biacore plots of the interaction between a mutant soluble A6 TCR (Clone 1) and HLA-A2 Tax.

Figures 32a and 32b show Biacore plots of the interaction between a mutant soluble A6 TCR (Clone 111) and HLA-A2 Tax.

Figures 33a and 33b show Biacore plots of the interaction between a mutant soluble A6 TCR (Clone 89) and HLA-A2 Tax.

Figure 34 shows a Biacore plot of the interaction between a mutant soluble A6 TCR (containing Clone 71 and Clone 134 mutations) and HLA-A2 Tax.

Figure 35 shows a Biacore plot of the interaction between a mutant soluble A6 TCR (containing Clone 1and βG102→A mutations) and HLA-A2 Tax.

Figure 36a to 36c show Biacore plots of the interaction between a mutant soluble A6 TCR (containing Clone 89 and Clone 134 mutations) and HLA-A2 Tax.

Figure 37a and 37b show Biacore plots of the interaction between a mutant soluble A6 TCR (containing Clone 71 and Clone 89 mutations) and HLA-A2 Tax.

Figure 38 details the β chain variable domain amino acid sequences of the following A6 TCR clones:
38a – Wild-type, 38b - Clone 134, 38c - Clone 89, 38d - Clone 1 and 38e - Clone 111

The mutated residues are shown in bold, bracketed residues are alternative residues that may be present at a particular site.

Figures 39A and 39B I

Example 1 – Design of primers and mutagenesis of A6 Tax TCR α and β chains to introduce the cysteine residues required for the formation of a novel inter-chain disulphide bond

For mutating A6 Tax threonine 48 of exon 1 in TRAC*01 to cysteine, the following primers were designed (mutation shown in lower case):

5'-C ACA GAC AAA tgT GTG CTA GAC AT **(SEQ ID 35)**
5'-AT GTC TAG CAC Aca TTT GTC TGT G **(SEQ ID 36)**

For mutating A6 Tax serine 57 of exon 1 in both TRBC1*01 and TRBC2*01 to cysteine, the following primers were designed (mutation shown in lower case):

5'-C AGT GGG GTC tGC ACA GAC CC **(SEQ ID 37)**
5'-GG GTC TGT Gca GAC CCC ACT G **(SEQ ID 38)**

PCR mutagenesis:
Expression plasmids containing the genes for the A6 Tax TCR α or β chain were mutated using the α-chain primers or the β-chain primers respectively, as follows. 100 ng of plasmid was mixed with 5 µl 10 mM dNTP, 25 µl 10xPfu-buffer (Stratagene), 10 units Pfu polymerase (Stratagene) and the final volume was adjusted to 240 µl with H_2O. 48 µl of this mix was supplemented with primers diluted to give a final concentration of 0.2 µM in 50 µl final reaction volume. After an initial denaturation step of 30 seconds at 95°C, the reaction mixture was subjected to 15 rounds of denaturation (95°C, 30 sec.), annealing (55°C, 60 sec.), and elongation (73°C, 8 min.) in a Hybaid PCR express PCR machine. The product was then digested

for 5 hours at 37°C with 10 units of DpnI restriction enzyme (New England Biolabs). 10 µl of the digested reaction was transformed into competent XL1-Blue bacteria and grown for 18 hours at 37°C. A single colony was picked and grown over night in 5 ml TYP + ampicillin (16 g/l Bacto-Tryptone, 16 g/l Yeast Extract, 5 g/l NaCl, 2.5 g/l K_2HPO_4, 100 mg/l Ampicillin). Plasmid DNA was purified on a Qiagen mini-prep column according to the manufacturer's instructions and the sequence was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University. The respective mutated nucleic acid and amino acid sequences are shown in Figures 1a and 2a for the α chain and Figures 1b and 2b for the β chain.

Example 2 – Construction of phage display vectors and cloning of A6 TCR α and β chains into the phagemid vectors.

In order to display a heterodimeric A6 TCR containing a non-native disulfide inter-chain bond on filamentous phage particles, phagemid vectors were constructed for expression of fusion proteins comprising the heterodimeric A6 TCR containing a non-native disulfide inter-chain bond with a phage coat protein. These vectors contain a pUC19 origin, an M13 origin, a bla (Ampicillin resistant) gene, Lac promoter/operator and a CAP-binding site. The design of these vectors is outlined in Figure 3, which describes vectors encoding for both the A6 TCR β chain-gp3 or A6 TCR β chain-gp8 fusion proteins in addition to the soluble A6 TCR α chain. The expression vectors containing the DNA sequences of the mutated A6 TCR α and β chains incorporating the additional cysteine residues required for the formation of a novel disulfide inter-chain bond prepared in Example 1 and as shown in figures 1a and 1b were used as the source of the A6 TCR α and β chains for the production of a phagemid encoding this TCR. The complete DNA sequence of the phagemid construct (pEX746) utilised is given in Figure 4.

The molecular cloning methods for constructing the vectors are described in "Molecular cloning: A laboratory manual, by J. Sambrook and D. W. Russell".

Primers listed in table-1 are used for construction of the vectors. A example of the PCR programme is 1 cycle of 94°C for 2 minutes, followed by 25 cycles of 94°C for 5 seconds, 53°C for 5 seconds and 72°C for 90 seconds, followed by 1 cycles of 72°C for 10 minutes, and then hold at 4°C. The Expand hifidelity Taq DNA polymerase is purchased from Roche.

Table 1. Primers used for construction of the A6 TCR phage display vectors

Primer name	Sequence 5′ to 3′
YOL1	TAATAATACGTATAATAATATTCTATTTCAAG GAGACAGTC **(SEQ ID 39)**
YOL2	CAATCCAGCGGCTGCCGTAGGCAATAGGTATT TCATTATGACTGTCTCCTTGAAATAG **(SEQ ID 40)**
YOL3	CtaCGGCAGCCGCTGGATTGTTATTACTCGCG GCCCAGCCGGCCATGGCccag **(SEQ ID 41)**
YOL4	GTTCTGCTCCACTTCCTTCTGGGCCATGGCCG GCTGGGCCG **(SEQ ID 42)**
YOL5	CAGAAGGAAGTGGAGCAGAAC **(SEQ ID 43)**
YOL6	CTTCTTAAAGAATTCTTAATTAACCTAGGTTA TTAGGAACTTTCTGGGCTGGGGAAG **(SEQ ID 44)**
YOL7	GTTAATTAAGAATTCTTTAAGAAGGAGATATA CATATGAAAAAATTATTATTCGCAATTC **(SEQ ID 45)**
YOL8	CGCGCTGTGAGAATAGAAAGGAACAACTAAAG GAATTGCGAATAATAATTTTTTCATATG **(SEQ ID 46)**
YOL9	CTTTCTATTCTCACAGCGCGCAGGCTGGTGTC ACTCAGAC **(SEQ ID 47)**

YOL10	ATGATGTCTAGATGCGGCCGCGTCTGCTCTAC CCCAGGCCTC **(SEQ ID 48)**
YOL11	GCATCTAGACATCATCACCATCATCACTAGAC TGTTGAAAGTTGTTTAGCAAAAC **(SEQ ID 49)**
YOL12	CTAGAGGGTACCTTATTAAGACTCCTTATTAC GCAGTATG **(SEQ ID 50)**

Example 3 – Expression of fusions of bacterial coat protein and heterodimeric A6 TCR in E. coli.

In order to validate the construct made in Example 2, phage particles displaying the heterodimeric A6 TCR containing a non-native disulfide inter-chain bond were prepared using methods described previously for the generation of phage particles displaying antibody scFvs (Li *et al*, 2000, Journal of Immunological Methods **236:** 133-146) with the following modifications. *E. coli* XL-1-Blue cells containing pEX746:A6 phagemid (i.e. the phagemid encoding the soluble A6 TCR α chain and an A6 TCR β chain fused to the phage gIII protein produced as described in Example 2) were used to inoculate 5 ml of Lbatg (Lennox L broth containing 100μg/ml of ampicillin, 12.5 μg/ml tetracycline and 2% glucose), and then the culture was incubated with shaking at 37°C overnight (16 hours). 50 μl of the overnight culture was used to inoculate 5 ml of TYPatg (TYP is 16g/l of peptone, 16g/l of yeast extract, 5g/l of NaCl and 2.5g/l of K_2HPO_4), and then the culture was incubated with shaking at 37°C until OD_{600nm} = 0.8. Helper phage M13 K07 was added to the culture to the final concentration of 5 X 10^9 pfu/ml. The culture was then incubated at 37°C stationary for thirty minutes and then with shaking at 200 rpm for further 30 minutes. The medium of above culture was then changed to TYPak (TYP containing 100μg/ml of ampicillin, 25 μg/ml of kanamycin), the culture was then incubated at 25°C with shaking at 250 rpm for 36 to 48 hours. The culture was then centrifuged at 4°C for 30 minutes at 4000 rpm. The supernatant was filtrated through a 0.45 μm syringe filter and stored at 4°C for further concentration or analysis.

The fusion protein of filamentous coat protein and heterodimeric A6 TCR containing a non-native disulfide inter-chain bond was detected in the supernatant by western blotting. Approximately 10^{11} cfu phage particles were loaded on each lane of an SDS-PAGE gel in both reducing and non-reducing loading buffer. Separated proteins were primary-antibody probed with an anti-M13 gIII mAb, followed by a second antibody conjugated with Horseradish Peroxidase (HRP). The HRP activity was then detected with Opti-4CN substrate kit from Bio-Rad (Figure 5). Theses data indicated that disulfide-bonded A6 TCR of clone 1 is fused with filamentous phage coat protein, gIII protein.

Example 4 – Detection of functional heterodimeric A6 TCR containing a non-native disulfide inter-chain bond on filamentous phage particles

The presence of functional (HLA-A2-tax binding) A6 TCR displayed on the phage particles was detected using a phage ELISA method.

TCR-Phage ELISA
Binding of the A6 TCR-displaying phage particles to immobilised peptide-MHC in ELISA is detected with primary rabbit anti-fd antisera (Sigma) followed by alkaline phosphatase (AP) conjugated anti-Rabbit mAb (Sigma). Non specific protein binding sites in the plates can be blocked with 2% MPBS or 3% BSA-PBS

Materials and reagents

1. Coating buffer, PBS
2. PBS: 138mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4
3. MPBS , 3% marvel-PBS
4. PBS-Tween: PBS, 0.1% Tween-20
5. Substrate solution, Sigma FAST pNPP, Cat# N2770

Method

1. Rinse NeutrAvidin coated wells twice with PBS.

2. Add 25µl of biotin-HLA-A2 Tax or biotin-HLA-A2 NYESO in PBS at concentration of 10 µg/ml, and incubate at room temperature for 30 to 60 min.

3. Rinse the wells twice with PBS

4. Add 300 µl of 3% Marvel-PBS, and incubate at room temperature for 1hr. Mix the TCR-phage suspension with 1 volume of 3% Marvel-PBS and incubate at room temperature.

5. Rinse the wells twice with PBS

6. Add 25 µl of the mixture of phage-A6 TCR/Marvel-PBS, incubate on ice for 1hr

7. Rinse the wells three times with ice-cold PBStween, and three times with ice-cold PBS.

8. Add 25 µl of ice cold rabbit anti-fd antibody diluted 1:1000 in Marvel-PBS, and incubate on ice for 1hr

9. Rinse the wells three times with ice-cold PBStween, and three times with ice-cold PBS.

10. Add 25 µl of ice cold anti-rabbit mAb-Ap conjugate diluted 1:50,000 in Marvel-PBS, and incubate on ice for 1hr

11. Rinse the wells three times with ice-cold PBStween, and three times with ice-cold PBS.

12. Add 150 µl of Alkaline phosphatase yellow to each well and read the signal at 405nm

The results presented in Figure 6 indicate clone 1 produced a phage particle displaying an A6 TCR that can bind specifically to its cognate pMHC. (HLA-A2 Tax)

Analysis of the DNA sequence of this displayed A6 TCR revealed the presence of an 'opal' stop codon in the TCR β chain not present in the corresponding sequence of the expression vector construct of Example 2. This codon is 'read-through' with low frequency by ribosomes of the *E.coli* strain utilised resulting in the insertion of a tryptophan residue at this site and a much-reduced overall level of full-length β chain expression. From this observation it was inferred that only cells expressing this mutated A6 TCR sequence had survived the culture rounds of Example 3, and that therefore the high levels of A6 TCR predicted to be expressed by the original expression vector were toxic to the host cells.

Example 5 – single- chain TCR (scTCR) ribosome display

Construction of Ribosome display scTCR vectors for use in generation of ribosome display PCR templates.

Ribosome display constructs were cloned into the readily available DNA plasmid pUC19 in order to generate an error free and stable DNA PCR template from which to conduct subsequent ribosome display experiments. Vector construction was undertaken in two steps so as to avoid the use of large oligonucleotide primers (with their associated error problems). The final A6 scTCR-C-Kappa DNA ribosome display construct is shown in a schematic form in figure 7a and both DNA and protein sequences are shown in Figure 7b. This construct can be excised from pUC19 as a Pst1/EcoR1 double digest.

The molecular cloning methods for constructing the vectors are described in "Molecular cloning: A laboratory manual, by J. Sambrook and D. W. Russell". Primers listed in Table 2 are used for construction of the vectors. The PCR programme utilised was as follows – 1 cycle of 94°C for 2 minutes, followed by 25 cycles of 94°C for 30 seconds, 55°C for 20 seconds and 72°C for 120 seconds, followed by 1 cycles of 72°C for 5 minutes, and then hold at 4°C. The Pfu DNA polymerase is purchased from Strategene. Oligonucleotide primers used are described in table 2.

Construction of pUC19-T7- Step 1

The construction of pUC19-T7 is described below, the construction results in a pUC19 vector containing a T7 promoter region followed by a short space region and the an optimum eukaryotic Kozak sequence. This is an essential part of the ribosome display construct as it is required for the initiation of transcription of any attached sequence in rabbit reticulocyte lysates. Sequences for ribosome display such as the A6scTCR-Ckappa can be ligated into the pUC19-T7 vector between the Nco1 and EcoR1 restriction sites.

Equimolar amounts of the primer Rev-link and For-link were annealed by heating to 94°C for 10 min and slowly cooling the reaction to room temperature. This results in the formation of a double stranded DNA complex that can be seen below.

5'AGCTGCAGCTAATACGACTCACTATAGGAACAGGCCACCATGG
CGTCGATTATGCTGAGTGATATCCTTGTCCGGTGGTACCCTAG 3'

(SEQ ID 51)

The 5' region contains an overhanging sticky end complimentary to a HindIII restriction site whilst the 3' end contains a sticky end that is complimentary to a BamH1 restriction site.

The annealed oligonucleotides were ligated into Hind III/BamHI double-digested pUC19 which had been purified by agarose gel electrophoresis, excised and further purified with the Qiagen gel extraction kit. The ligations were transformed into *E. coli* XL1-BLUE. Individual pUC19-T7 clones were sequenced to confirm the presence of the correct sequence. The sequence is shown in Figure 8.

Construction of A6scTCR-C-Kappa vector – Step 2.

Construction of the single chain A6scTCR-C-Kappa DNA sequence requires the generation of three PCR fragments that must then be assembled into one A6scTCR-C-Kappa fragment. The fragments consist of (a.) the A6 TCR alpha chain variable region flanked by a Nco1 site in the 5' region and a section of Glycine Serine linker in the 3' region flanked by a BamH1 restriction site. This product was generated via a standard PCR of the vector pEX202 with the primers 45 and 50 (See Table 2). Fragment (b.) A6 TCR beta variable and constant region flanked by a BamH1 restriction site in the 5' region followed by a section of Glycine Serine linker. This product was generated via a standard PCR of the vector pEX207 with the primers 72 and 73 (See Table 2). Fragment (c.) Portion of a human C-kappa region generated by a standard PCR of the p147 vector with the primers 61-60 (See Table 2). All PCR products were run on a 1.6% TBE agarose gel and DNA bands of the correct size excised and purified using the Qiagen gel extraction kit.

Fragments (b.) and (c.) were fused by a standard overlap PCR via the complementarity in their primer sequences 73 and 61(See Table 2). The PCR was carried out via the primers 72 and 60 (See Table 2). The PCR products were run on a 1.6% TBE agarose gel and DNA bands of the correct size excised and purified using the Qiagen gel extraction kit. This fragment is termed (d.).

Fragment (a.) was double digested with Nco1 and BamH1 whilst fragment (d.) was double digested with BamH1 and EcoR1. pUC19-T7 was double digested with Nco1 and EcoR1. All digested DNA products were run on a 1.2% TBE agarose gel and DNA bands of the correct size were excised and purified using the Qiagen gel extraction kit. The digested pUC19-T7, fragments (a.) and (d.) were ligated and transformed into E. coli XL1-BLUE. Transformants were sequenced to confirm the correct sequence. The sequence of the A6scTCR-C-Kappa ribosome display construct that was cloned into pUC19 is shown in Figure 9 flanked by its Pst1 and EcoR1 sites.

Table 2.

Oligonucleotides used (Purchased from MWG).

Rev-Link	**5'** GATCCCATGGTGGCCTGTTCCTATAGTGAGTCGTATTAGCTGC **(SEQ ID 52)**
For-Link	**5'** AGCTGCAGCTAATACGACTCACTATAGGAACAGGCCACCATGG **(SEQ ID 53)**
45-A6	**5'** CCACCATGGGCCAGAAGGAAGTGGAGCAGAACTC **(SEQ ID 54)**
7 A6-Beta(RT-PCR)(a)	**5'** CGAGAGCCCGTAGAACTGGACTTG **(SEQ ID 55)**
49-A6-BamH1-F	**5'** GTGGATCCGGCGGTGGCGGGTCGAACGCTGGTGTCA CTCAGACCCC **(SEQ ID 56)**
50-A6-BamH1-R	**5'** CCGGATCCACCTCCGCCTGAACCGCCTCCACCGGTGACCACAAC CTGGGTCCCTG **(SEQ ID 57)**
60-Kappa-rev-EcoR1	**5'** CTGAGAATTCTTATGACTCTCCGCGGTTGAAGCTC **(SEQ ID 58)**
61-Betac-Kappa-for1	**5'** TGACGAATTCTGACTCTCCGCGGTTGAAGCTC **(SEQ ID 59)**
71 T7-Primer	**5'** AGCTGCAGCTAATACGACTCACTATAGG **(SEQ ID 60)**
72 A6-beta	**5'** GGCCACCATGGGCAACGCTGGTGTCACTCAGACCCC **(SEQ ID 61)**
73-A6-cons-rev	5' TGAACCGCCTCCACCGTCTGCTCTACCCCAGGCCTCGGCG **(SEQ ID 62)**
75 Kappa-rev	5' TGACTCTCCGCGGTTGAAGCTC **(SEQ ID 63)**

Demonstration of the production of sc A6 TCR-C-Kappa by In vitro transcription translation.

Preparation of scA6 TCR-C-Kappa PCR product for In vitro transcription translation.

Here we describe the synthesis of sc A6 TCR-C-Kappa via *In vitro* transcription translation in the presence of biotinylated lysine and its subsequent detection by western blotting and detection with alkaline phosphatase labelled streptavidin.

The sc A6 TCR-C-Kappa PCR product was prepared in a standard PCR reaction using the vector sc A6 TCR-C-Kappa as template and PCR primers 71 and 60. Primer 60 contains a stop codon to allow the release of the scTCR from the ribosome. Pfu polymerase (Strategene) was used for increased fidelity during PCR synthesis. The PCR products were run on a 1.6% TBE agarose gel and DNA bands of the correct size excised and purified using the Qiagen gel extraction kit.
The transcription translation reactions were carried out using the Ambion PROTEINscript II Linked transcription translation kit Cat 1280-1287 with 300ng of the above described PCR product. Three transcription translation reactions were set up according to the manufactures protocol. The one modification was the addition of biotinylated lysine from the Transcend™ Non-Radioactive Translation Detection System.

Reaction 1 sc A6 TCR-C-Kappa 300ng with 2µl biotinylated lysine
Reaction 2 sc A6 TCR-C-Kappa 300ng without 2µl biotinylated lysine
Reaction 3 No DNA control with 2µl biotinylated lysine.

Two microliters of each reaction was run on a 4-20% Novex gradient SDS-PAGE gel (Invitrogen). Additionaly a number of dilutions of a control biotinylated TCR were also run. The gel was blotted and the proteins detected with streptavidin alkaline phosphatase and subsequently colometrically developed with Western Blue ® Stabilized Substrate for Alkaline Phosphatase as described in the Transcend™ Non-Radioactive Translation Detection System protocol. The western blot is shown in Figure 10.

In the no DNA control and A6scTCR-C-Kappa reaction without biotinylated lysine no band of approximately the correct size can be seen as expected whilst in the A6scTCR-C-Kappa reaction in the presence of biotinylated lysine a band of approximately the correct size can be seen. This demonstrates the synthesis of the sc A6 TCR-C-Kappa TCR by *In vitro* transcription translation.

Preparation of sc A6 TCR-C-Kappa ribosome display PCR product.

The sc A6 TCR-C-Kappa PCR product was prepared in a standard PCR reaction using the vector A6scTCR-C-Kappa as template and PCR primers 71 and 75 (See Table 2). Primer 75 does not contain a stop codon. Pfu polymerase (Strategene) was used for increased fidelity during PCR synthesis. The PCR products were run on a 1.6% TBE agarose gel and DNA bands of the correct size were excised and purified using the Qiagen gel extraction kit.

Ribosome Display Process
Transcription and translation of sc A6 TCR-C-Kappa

The transcription / translation reactions were carried out using the Ambion PROTEINscript II Linked transcription translation kit (Cat No. 1280-1287)

Transcription reactions

The following transcription reactions were set up in Ambion 0.5 ml non stick tubes (Cat No. 12350).

Contents	Tube 1 **(Normal A6)**	Tube 2 **(Control)**
Water	4.53 µl	5.7µl
Template (PCR product)	**Sc A6 TCR-C-Kappa PCR product 1.17µl (300 ng)**	No DNA
5X transcription mix	2 µl	2µl
Enzyme mix	2µl	2µl
Superasin Rnase inhibitor ambion	0.3µl	0.3µl
Final volume	10µl	10µl

The tubes were incubated at 30°C for 60 min on a PCR block with the hot lid off.

Translation reactions

The following translation reactions were set up in Ambion 0.5 ml non stick tubes.

Contents	1 **(Normal A6)**	2 **(Control)**
Reticulocyte Lysate	105µl	105 µl
25mM Mg-Acetate	3µl	3 µl
Translation Mix	7.5µl	7.5 µl
Methionine	7.5µl	7.5 µl
Water	18µl	18 µl
Superasin Rnase inhibitor	3µl	3 µl
Transcription reaction	6µl tube 1 above	6µl tube 2 above

Each tube contains enough for 3x50µl selections. The tubes were mixed and incubated at 30°C for 60 min on a PCR block with the hot lid off. After 30 min 3 Unit of RQ1 Rnase free Dnase (Promega) was added to destroy the original DNA template in tube 1

and 3 Unit RQ1 Rnase free Dnase (Promega) in tube 2. After 60 min 18µl of Heparin solution was added to translation reaction 2 and 18µl of Heparin solution was added to translation reaction 1. Samples were stored on ice ready for selection against HLA-coated beads.

Coating of magnetic beads.

20µl of resuspended Streptavidin Magnetic Particles (Roche Cat. No. 1641778) were transferred into a sterile Rnase free 1.5 ml eppendorf tube. The beads were immobilised with a Magnetic Particle Separator (Roche Cat. No. 1641794) and the supernatant was removed. The beads were then washed with 100 µl of Rnase free 1 X PBS (10 x PBS Ambion Cat No. 9624, Ambion H_2O Cat No. 9930) the beads were immobilised and the supernatant was removed. A total of 3 PBS washes were carried out.

The beads were resuspended in 20µl of PBS and the contents split evenly between two tubes (10µl each). One tube will be used to produce control-blocked beads and the other tubes to produce HLA-A2-Tax coated beads.

To the control beads tube 80µl of BSA/Biotin solution was added and mixed. The BSA/Biotin solution was made up as follows. 10µl of a 0.2M Tris base 0.1M Biotin solution was added to 990µl of PBS 0.1 % BSA (Ambion Ultrapure Cat No. 2616). Also 20 µl of Heparin solution (138 mg/ml Heparin (Sigma H-3393) in 1 x PBS) was added and the solution mixed. The beads were incubated at room temperature for 1 hour with intermittent mixing. The beads were then washed three times with 100µl of PBS and were resuspended in 10 µl of PBS, 0.1% BSA.

The HLA-TAX coated beads were prepared as follows. 40 µl of HLA-A2-Tax(1.15 mg/ml prepared as described in WO99/60120) was added to the 10 µl of beads and mixed. The beads were incubated at room temperature for 15 min and then 20 µl of BSA 50mg/ml Ambion Cat 2616 and 20 µl of heparin solution (see above) were added and mixed. The beads were incubated for a further 45 min and then 20 µl of

BSA/Biotin solution was added. The beads were then washed three times with 100 µl of PBS and were re-suspended in 10 µl of PBS, 0.1% BSA.

Panning with magnetic beads

The sc A6 TCR translation reaction was split into three 50µl aliquots and each aliquot received either 2µl of the following beads:

Control (no HLA)
HLA-A2-Tax
HLA-A2-Tax plus 10µg soluble scA6 TCR

A control translation reaction was also carried out and split into three 50µl aliquots and each aliquot received either 2µl of the following beads

Control (no HLA)
HLA-A2-Tax
HLA-A2-Tax plus 10µg soluble sc A6 TCR

This gave a total of six tubes. The tubes were incubated on a PCR block at 5°C for 60 min with intermittent mixing.
The beads were then washed three times with 100µl ice cold buffer (PBS, 5mM Mg-acetate, 0.2% Tween 20(Sigma Rnase free). Each aliquot of beads were then re-suspended in 50µl of 1 x RQ1 Dnase digestion buffer containing 1µl (40 U) of Superasin and 1µl (1U) of RQ1 Dnase. The beads were incubated on a PCR block for 30 min at 30°C.

The beads were then washed three times with 100µl ice cold buffer (PBS, 5mM Mg-acetate, 0.2% Tween 20) and once with ice cold H_2O. The beads were re-suspended in 10µl of Rnase free H_2O. The beads were then frozen ready for RT-PCR.

RT-PCR of sc A6 TCR-C-Kappa mRNA on beads rescued from the ribosome display reactions.

The RT PCR reactions on the beads were carried out using the Titan one tube RT-PCR kit cat 1855476 as described in the manufacturers protocols. Two microliters of beads were added into each RT-PCR reaction along with the primers 45 and 7 and 0.3µl of Superasin Rnase inhibitor.

For each RT-PCR reaction a second PCR only reaction was set up which differed only by the fact that no reverse transcriptase was present just Roche high fidelity polymerase. This second reaction served as a control for DNA contamination. Additionally a RT-PCR positive control control was set up using 1ng of the vector sc A6 TCR-C-Kappa.

The reactions were cycled as follows. An RT-PCR step was carried out by incubation of the samples at 50°C for 30 min followed by the inactivation of the reverse transcriptase by incubation at 94°C for 3 min on a PCR block.

The reactions were PCR cycles as follows for a total of 38 cycles:

94°C 30 seconds
55°C 20 seconds
68°C 130 seconds.

The PCR reaction was finished by incubation at 72°C for 4 minutes.

Great care was taken during all ribosome display steps to avoid Rnase contamination. The RT-PCR and PCR reactions were run on a 1.6% TBE agarose gel which can be seen in Figure 11. Analysis of the gel shows that there is no DNA contamination and that all PCR products are derived from mRNA. The DNA band of the correct size in lane 2 demonstrates that ribosome displayed sc A6 TCR-C-Kappa was selected out by HLA-A2-Tax coated beads. Lane 3 shows that we can inhibit this specific selection of ribosome-displayed sc A6 TCR-C-Kappa by the addition of soluble sc A6 TCR. The significant reduction in the band intensity in lane 3 relative to the uninhibited sample

in lane 2 demonstrates this. No binding of ribosome-displayed sc A6 TCR-C-Kappa could be shown against control non-HLA coated beads.

Example 6 – Sequence Analysis of A6 TCR clones displayed on phage particles and methods to improve display characteristics

After the construction of vectors for displaying A6 TCR on phage by PCR and molecular cloning, bacterial clones that can produce phage particles displaying A6 TCR were screened by phage ELISA as described in Example 4. Three different clones were identified that gave specific binding to HLA-A2-tax in the ELISA binding assay. These clones all contained mutations in the 'wild-type' A6 TCR DNA or in the associated regulatory sequences, which are described in the following table:

Functional clones from screening TCR A6 displayed on phage

Name	Feature	
Clone 7	The third ribosome-binding site, which is located in front of vβ gene, is mutated from AAGGAGA to AAGGGGA.	
Clone 9	An opal codon is introduced in vβ CDR3.	Full DNA and amino acid sequence in Figures 12a & 12b
Clone 49	An amber codon is introduced in vβ FR1. This mutation introduces a 'silent' mutation that does not affect the resulting amino acid sequence	Full DNA sequence in Figures 13a

These clones all contained mutations that are likely to cause a reduction in the expression levels of the A6 TCR β chain. It was inferred that low expression clones were selected over high expression clones as a result of cell toxicity caused by high expression levels of TCR.

Example 7– Mutagenesis of A6 TCR CDR3 regions

The CDR3 regions of the A6 TCR were targeted for the introduction of mutations to investigate the possibility of generating high affinity mutants.

Overlapping PCR was used to modify the sequence of α and β CDR3 regions to introduce two unique restriction sites *Hind III* for α chain, with oligos of YOL54, 5'CAGCTGGGGGAAGCTTCAGTTTGGAGCAG3'**(SEQ ID 64)** and YOL55, 5'CTGCTCCAAACTGAAGCTTCCCCCAGCTG3'**(SEQ ID 65)**, and *Xho* I for β chain, with oligos of YOL56 5'GTACTTCTGTGCCTCGAGGCCGGGACTAG3' **(SEQ ID 66)** and YOL57 5'CTAGTCCCGGCCTCGAGGCACAGAAGTAC3' **(SEQ ID 67)**.

PCR was used to introduce mutations for affinity maturation. The A6 TCR clone 9 (incorporating an introduced opal codon in the β chain CDR3 sequence) was used as a source of template DNA, and TCR chains were amplified with the mutation primers (detailed in the following table) and YOL22 5'CATTTTCAGGGATAGCAAGC3' **(SEQ ID 68)** (β-chains) or YOL13 5'TCACACAGGAAACAGCTATG3' **(SEQ ID 69)**(α-chains).

Primers for introducing mutation at CDR3 of A6 β chain and α chain

Primer name	Sequence 5' to 3'
YOL59	TGTGCCTCGAGGNNKNNKNNKNNKNNKN NKCGACCAGAGCAGTACTTCG **(SEQ ID 70)**
YOL60	TGTGCCTCGAGGCCGNNKNNKNNKNNKN NKNNKCCAGAGCAGTACTTCGGGC **(SEQ ID 71)**
YOL61	TGTGCCTCGAGGCCGNNKNNKNNKNNKN NKNNKCGACCAGAGCAGTACTTCG **(SEQ ID 72)**
YOL62	TGTGCCTCGAGGCCGNNKNNKNNKNNKN NKNNKGGAGGGCGACCAGAGCAG **(SEQ ID 73)**

YOL63	TGTGCCTCGAGGCCGGGANNKNNKNNKN NKNNKNNKGGGCGACCAGAGCAGTAC **(SEQ ID 74)**
YOL68	TGTGCCTCGAGGNNKNNKNNKNNKNNKN NKCCAGAGCAGTACTTCGggc **(SEQ ID 75)**
YOL69	TGTGCCTCGAGGNNKNNKNNKNNKNNKN NKGAGCAGTACTTCGggccg **(SEQ ID 76)**
YOL70	TGTGCCTCGAGGNNKNNKNNKNNKNNKN NKCAGTACTTCGggccgggc **(SEQ ID 77)**
YOL71	TGTGCCTCGAGGccgNNKNNKNNKNNKg ggCGACCAGAGCAGTACTTCG **(SEQ ID 78)**
YOL58	AAACTGAAGCTTMNNMNNMNNMNNMNNT GTAACGGCACAGAGGTAG **(SEQ ID 79)**
YOL72	AAACTGAAGCTTMNNMNNgctgtcMNNT GTAACGGCACAGAGGTAG **(SEQ ID 80)**
YOL73	AAACTGAAGCTTMNNMNNMNNgctgtcM NNTGTAACGGCACAGAGGTAG **(SEQ ID 81)**
YOL74	AAACTGAAGCTTMNNMNNgctgtcMNNA ACGGCACAGAGGTAG **(SEQ ID 82)**

α-chain fragments were digested with *Nco I* and *HindIII* and re-purified using a Qiagen kit and vector was prepared by digesting clone 9 with *Nco I* and *HindIII* followed by gel purification using a Qiagen kit. β-chain fragments were digested with *Xho I* and *Not I* and re-purified using a Qiagen kit and vector was prepared by digesting clone 9 with *Xho I* and *Not I* followed by gel purification using a Qiagen kit. Purified inserts and vectors at 3:1 molar ratio were mixed with T4 ligase buffer, T4 ligase and nuclease-free water. The ligations were carried out at 16°C water bath overnight. For each mutation-library, a total of 0.5 to 1μg purified ligated products were electroporated into *E. coli* TG1 at ratio of 0.2μg DNA per 40 μl of electroporation-competent cells (Stratagen) following the protocols provided by the manufacturer. After electroporation, the cells were re-suspended immediately with 960μl of SOC medium at 37°C and plated on a 244mm x244mm tissue culture plate containing YTE (15g Bacto-Agar, 8g NaCl, 10g Tryptone, 5g Yeast Extract in 1 litre)

supplemented with 100μg/ml ampicillin and 2% glucose. The plate was incubated at 30°C over night. The cells were then scraped from the plates with 5 ml of DYT (16g Trytone, 10g Yeast extract and 5g NaCl in 1 litre, autoclaved at 125°C for 15 minutes) supplemented with 15% glycerol.

In order to make phage particles displaying the A6 TCR, 500 ml of DYTag (DYT containing 100 μg/ml of ampicillin and 2% glucose) was inoculated with 500 to 1000 μl of the library stocks. The culture was grown until OD(600nm) reached 0.5. 100 ml of the culture was infected with helper phage (M13 K07 (Invitrogen), or HYPER PHAGE (Progen Biotechnik, GmbH 69123 Heidelberg), and incubated at 37°C water bath for 30 minutes. The medium was replaced with 100 ml of DYTak (DYT containing 100 μg/ml ampicillin and 25 μg/ml of kanamycin). The culture was then incubated with shaking at 300 rpm and 25°C for 20 to 36 hours.

Example 8 – Isolation of high affinity A6 TCR mutants

The isolation of high affinity A6 TCR mutants was carried out using two different methods.

The first method involves selecting phage particles displaying mutant A6 TCRs capable of binding to HLA-A2 Tax complex using Maxisorp immuno-tubes (Invitrogen) The immuno-tubes were coated with 1 to 2 ml 10μg/ml streptavidin in PBS overnight at room temperature. The tubes were washed twice with PBS, and then 1 ml of biotinylated HLA-A2 Tax complex at 5μg/ml in PBS was added and incubated at room temperature for 30 minutes. The rest of the protocol for selection of high affinity binders is as described previously (Li *et al.* (2000) *Journal of Immunological Methods* 236: 133-146), except for the following modifications. The selection was performed over three or four rounds. The concentrations of biotinylated HLA-A2 Tax complex were 5μg/ml for the first round of selection, 0.5 μg/ml for the second, 0.05 μg/ml for the third and 0.005 μg/ml for the fourth round of selection. M13 K07 helper

phage were used in rounds one and two, and hyper phage were used in subsequent rounds, for the selection.

The second method utilised was the selection of phage particles displaying mutant A6 TCRs capable of binding to HLA-A2 Tax complex in solution. Streptavidin-coated paramagnetic beads (Dynal M280) were pre-washed according to manufacturer's protocols. Phage particles, displaying mutated A6 TCR at a concentration of 10^{12} to 10^{13} cfu, were pre-mixed with biotinylated HLA-A2 Tax complex at concentrations of $2x10^{-8}$M, $2x10^{-9}$M, $2x10^{-10}$M and $2x\ 10^{-11}$M for first, second, third and fourth-round of selections respectively. The mixture of A6 TCR-displaying phage particles and HLA-A2 Tax complex was incubated for one hour at room temperature with gentle rotation, and A6 TCR-displaying phage particles bound to biotinylated HLA-A2 Tax complex were captured using 200 µl (round 1) or 50µl (round 2, 3, and 4) of streptavidin-coated M280 magnetic beads. After capture of the phage particles, the beads were washed a total of ten times (three times in PBStween20, twice in PBStween 20 containing 2% skimmed milk powder, twice in PBS, once in PBS containing 2% skimmed milk powder, and twice in PBS) using a Dynal magnetic particle concentrator. After final wash, the beads was re-suspended in 1ml of freshly prepared 100mM triethylamine pH11.5, and incubated for 5 to 10 minutes at room temperature with gentle rotation. Phage particles eluted from the beads were neutralized immediately with 300 µl of 1M tris-HCl pH7.0. Half of the eluate was used to infect 10 ml of *E.coli* TG1 at OD(600nm)=0.5 freshly prepared for the amplification of the selected phage particles according to the methods previously described (Li *et al.*, (2000) *Journal of Immunological Methods* 236: 133-146).

After the third or fourth round of selection, 95 colonies were picked from the plates and used to inoculate 100 µl of DYTag in a 96-well microtiter plate. The culture was incubated at 37°C with shaking overnight. 100 µl of DYTag was then sub-inoculated with 2 to 5 µl of the overnight cultures, and incubated at 37°C with shaking for 2 to 3 hours or until the culture became cloudy. To infect the cells with helper phage, the culture was infected with 25 µl of DYTag containing 5 x 10^9 pfu helper phages, and

incubated at 37°C for 30 minutes. The medium was replaced with DYTak. The plates were incubated at 25°C for 20 to 36 hours with shaking at 300 rpm. The cells were precipitated by centrifugation at 3000g for 10 minutes at 4°C. Supernatants were used to screen for high affinity A6 TCR mutants by competitive phage ELISA as follows.

Nunc-Immuno Maxisorp wells coated with streptavidin were rinsed twice with PBS. 25 µl 5 µg/ml biotinylated HLA-A2-Tax complex was added to each well and these were incubated at room temperature for 30 to 60 minutes, and followed by two PBS rinses. Non-specific protein binding sites in the wells were blocked by the addition of 300 µl 3% skimmed milk in PBS followed by incubation at room temperature for 2 hours. In order to prepare phage particles displaying the heterodimric A6 TCR, phage particles were mixed with 3% skimmed milk in PBS containing 0, 20, and 200 nM HLA-A2-Tax, followed by incubated at room temperature for 1 hour. The phage is added to the wells coated with HLA-A2-Tax and incubated at room temperature for 1 hour, followed by 3 washes with PBS containing 0.1% tween 20 and then 3 washes with PBS. The bound TCR-dispalying phage particles are detected with an anti-fd antibody (Sigma) as described in Example 4.

Several putative high affinity A6 TCR mutants were identified, and the CDR3 sequences are listed in the two following tables along with the corresponsding wild-type sequences. Amber stop codons (**X**) were found in all β chain mutants and one α chain mutant.

A6 TCR β chain mutants

clone	CDR3 sequence
Wild Type	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCAGAGCAGTAG **(SEQ ID 83)** A S R P G L A G G R P E Q Y **(SEQ ID 84)**
134	GCCTCGAGGCCGGGGCTGATGAGTGCGTAGCCAGAGCAGTAC **(SEQ ID 85)** A S R P G L **M S A X** P E Q Y **(SEQ ID 86)**

86	GCCTCGAGGCCGGGGCTGAGGTCGGCGTAGCCAGAGCAGTAC **(SEQ ID 87)** A S R P G L **R S A X** P E Q Y **(SEQ ID 88)**
87	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCAGAGGCGTAG **(SEQ ID 89)** A S R P G L A G G R P E **A X** **(SEQ ID 90)**
89	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCAGAGGATTAG **(SEQ ID 91)** A S R P G L A G G R P E **D X** **(SEQ ID 92)**
85	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCAGATCAGTAG **(SEQ ID 93)** A S R P G L A G G R P **D** Q **X** **(SEQ ID 94)**
83	GCCTCGAGGCCGGGTCTGTAGGCTGGGCGACCAGAGCAGTAC **(SEQ ID 95)** A S R P G L **X A G** R P **E** Q Y **(SEQ ID 96)**
1	GCCTCGAGGCCGGGGCTGGTTCCGGGGCGACCAGAGCAGTAG **(SEQ ID 97)** A S R P G L **V P** G R P E Q **X** **(SEQ ID 98)**
2	GCCTCGAGGCCGGGGCTTGTGTCTGCTTAGCCAGAGCAGTAC **(SEQ ID 99)** A S R P G L **V S A X** P E Q Y **(SEQ ID 100)**
111	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCACATCCGTAG **(SEQ ID 101)** A S R P G L A G G R P **H P X** **(SEQ ID 102)**
125	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCAGATGCGTAG **(SEQ ID 103)** A S R P G L A G G R P **D A X** **(SEQ ID 104)**
133	GCCTCGAGGCCGGGTCTGATTAGTGCTTAGCCAGAGCAGTAC **(SEQ ID 105)** A S R P G L **I S A X** P E Q Y **(SEQ ID 106)**

5

A6 TCR α chain mutants

Clone	CDR3
Wild Type	GCCGTTACAACTGACAGCTGGGGGAAGCTTCAG **(SEQ ID 107)** A V T T D S W G K L Q **(SEQ ID 108)**
149	GCCGTTACAACTGACAGCTGGGGGCCGCTTCAG **(SEQ ID 109)** A V T T D S W G **P** L Q **(SEQ ID 110)**
65	GCCGTTACAACTGACAGCTGGGGGAAGATGCAG **(SEQ ID 111)** A V T T D S W G K **M** Q **(SEQ ID 112)**
66	GCCGTTACAACTGACAGCTGGGGGAAGTTGCAT **(SEQ ID 113)** A V T T D S W G K L **H** **(SEQ ID 114)**
153	GCCGTTACAACTGACAGCTGGGGGTAGCTTCAT **(SEQ ID 115)** A V T T D S W G **X** L **H** **(SEQ ID 116)**
71	GCCGTTACAACTGACAGCTGGGGGGAGCTTCAT **(SEQ ID 117)** A V T T D S W G **E** L **H** **(SEQ ID 118)**
70	GCCGTTACAACTGACAGCTGGGGGAGGCTGCAT **(SEQ ID 119)** A V T T D S W G **R** L **H** **(SEQ ID 120)**
121	GCCGTTACAACTGACAGCTGGGGGCAGCTTCAT **(SEQ ID 121)** A V T T D S W G **Q** L **H** **(SEQ ID 122)**
117	GCCGTTACAACTGACAGCTGGGGGAAGGTTCAT **(SEQ ID 123)** A V T T D S W G K **V** **H** **(SEQ ID 124)**
72	GCCGTTACAACTGACAGCTGGGGGAAGGTGAAT **(SEQ ID 125)** A V T T D S W G K **V** **N** **(SEQ ID 126)**
150	GCCGTTACAACTGACAGCTGGGGGAAGCTTCTG **(SEQ ID 127)** A V T T D S W G K L **L** **(SEQ ID 128)**

Example 9 – Production of soluble heterodimeric A6 TCR with non-native disulfide bond between constant regions, containing CDR3 mutations

Phagemid DNA encoding the high affinity A6 TCR mutants identified in Example 8 was isolated from the relevant *E.coli* cells using a Mini-Prep kit (Quiagen, UK)

PCR amplification using the phagemid DNA as a target and the following primers was used to amplify the soluble TCR α and β chain DNA sequences.

A6 TCR alpha chain forward primer
ggaattc atcgatg cagaaggaagtggagcag **(SEQ ID 129)**
ClaI restriction site is underlined)

Universal TCR alpha chain reverse primer
gtacacggccgggtcagggttctggatatac **(SEQ ID 130)**
(EagI restriction site is underlined)

A6 beta chain forward primer
Tctctcattaatgaatgctggtgtcactcagacccc **(SEQ ID 131)**
(AseI restriction site is underlined)

Universal beta chain reverse primer
Tagaaaccggtggccaggcacaccagtgtggc **(SEQ ID 132)**
(AgeI restriction site is underlined)

In the case of the TCR β chain a further PCR stitching was carried out to replace the amber stop codon in the CDR3 region with a codon encoding glutamic acid. When an amber stop codon is suppressed in *E.coli*, a glutamine residue is normally introduced instead of the translation being stopped. Therefore, when the amber codon-containing TCR is displayed on the surface of phage, it contains a glutamine residue in this position. However, when the TCR β –chain gene was transferred into the expression plasmid, a glutamic acid residue was used as an alternative to glutamine. The primers used for this PCR stitching were as follows.

YOL124 CTGCTCTGGTTCCGCACTC
(SEQ ID 133)

YOL125 GAGTGCGGAACCAGAGCAG

(SEQ ID 134)

The DNA sequence of the mutated soluble A6 TCR β chain was verified by automated sequencing (see Figure 14a for the mutated A6 TCR β chain DNA sequence and 14b for the amino acid sequence encoded thereby). Figure 14c shows the mutated A6 TCR β chain amino acid sequence without the glutamine to glutamic acid substitution, i.e. the sequence that was present in Clone 134 as isolated by phage-ELISA.

Theses A6 TCR α and β DNA sequences were then used to produce a soluble A6 TCR as described in WO 03/020763. Briefly, the two chains are expressed as inclusion bodies in separate *E.coli* cultures. The inclusion bodies are then isolated, de-natured and re-folded together *in vitro*.

Example 10 – BIAcore surface plasmon resonance characterisation of a high affinity A6 TCR binding to HLA-A2 Tax.

A surface plasmon resonance biosensor (BIAcore 3000™) was used to analyse the binding of the high affinity clone 134 A6 TCR (See Figures 15a & 15b for the full DNA and amino acid sequences of the mutated TCR β chain respectively) to the HLA-A2 Tax ligand. This was facilitated by producing pMHC complexes (described below) which were immobilised to a streptavidin-coated binding surface in a semi-oriented fashion, allowing efficient testing of the binding of a soluble T-cell receptor to up to four different pMHC (immobilised on separate flow cells) simultaneously. Manual injection of HLA complex allows the precise level of immobilised class I molecules to be manipulated easily.

Biotinylated class I HLA-A2 tax complexes were refolded *in vitro* from bacterially-expressed inclusion bodies containing the constituent subunit proteins and synthetic

peptide, followed by purification and *in vitro* enzymatic biotinylation (O'Callaghan *et al.* (1999) *Anal. Biochem.* **266**: 9-15). HLA-heavy chain was expressed with a C-terminal biotinylation tag which replaces the transmembrane and cytoplasmic domains of the protein in an appropriate construct. Inclusion body expression levels of ~75 mg/litre bacterial culture were obtained. The HLA light-chain or β2-microglobulin was also expressed as inclusion bodies in *E.coli* from an appropriate construct, at a level of ~500 mg/litre bacterial culture.

E. coli cells were lysed and inclusion bodies were purified to approximately 80% purity. Protein from inclusion bodies was denatured in 6 M guanidine-HCl, 50 mM Tris pH 8.1, 100 mM NaCl, 10 mM DTT, 10 mM EDTA, and was refolded at a concentration of 30 mg/litre heavy chain, 30 mg/litre β2m into 0.4 M L-Arginine-HCl, 100 mM Tris pH 8.1, 3.7 mM cystamine, mM cysteamine, 4 mg/ml peptide (e.g. tax 11-19), by addition of a single pulse of denatured protein into refold buffer at < 5°C. Refolding was allowed to reach completion at 4°C for at least 1 hour.

Buffer was exchanged by dialysis in 10 volumes of 10 mM Tris pH 8.1. Two changes of buffer were necessary to reduce the ionic strength of the solution sufficiently. The protein solution was then filtered through a 1.5μm cellulose acetate filter and loaded onto a POROS 50HQ anion exchange column (8 ml bed volume). Protein was eluted with a linear 0-500 mM NaCl gradient. HLA-A2-peptide complex eluted at approximately 250 mM NaCl, and peak fractions were collected, a cocktail of protease inhibitors (Calbiochem) was added and the fractions were chilled on ice.

Biotinylation tagged HLA-A2 complexes were buffer exchanged into 10 mM Tris pH 8.1, 5 mM NaCl using a Pharmacia fast desalting column equilibrated in the same buffer. Immediately upon elution, the protein-containing fractions were chilled on ice and protease inhibitor cocktail (Calbiochem) was added. Biotinylation reagents were then added: 1 mM biotin, 5 mM ATP (buffered to pH 8), 7.5 mM MgCl2, and 5 μg/ml BirA enzyme (purified according to O'Callaghan *et al.* (1999) *Anal. Biochem.* **266**: 9-15). The mixture was then allowed to incubate at room temperature overnight.

Biotinylated HLA-A2 complexes were purified using gel filtration chromatography. A Pharmacia Superdex 75 HR 10/30 column was pre-equilibrated with filtered PBS and 1 ml of the biotinylation reaction mixture was loaded and the column was developed with PBS at 0.5 ml/min. Biotinylated HLA-A2 complexes eluted as a single peak at approximately 15 ml. Fractions containing protein were pooled, chilled on ice, and protease inhibitor cocktail was added. Protein concentration was determined using a Coomassie-binding assay (PerBio) and aliquots of biotinylated HLA-A2 complexes were stored frozen at –20°C. Streptavidin was immobilised by standard amine coupling methods.

The interactions between the high affinity A6 Tax TCR containing a novel inter-chain bond and the HLA-A2 Tax complex or an irrelevant HLA-A2 NY-ESO combination, the production of which is described above, were analysed on a BIAcore 3000™ surface plasmon resonance (SPR) biosensor. SPR measures changes in refractive index expressed in response units (RU) near a sensor surface within a small flow cell, a principle that can be used to detect receptor ligand interactions and to analyse their affinity and kinetic parameters. The probe flow cells were prepared by immobilising the individual HLA-A2 peptide complexes in separate flow cells via binding between the biotin cross linked onto β2m and streptavidin which have been chemically cross linked to the activated surface of the flow cells. The assay was then performed by passing sTCR over the surfaces of the different flow cells at a constant flow rate, measuring the SPR response in doing so. Initially, the specificity of the interaction was verified by passing soluble A6 TCR at a constant flow rate of 5 µl min-1 over four different surfaces; one coated with ~1000 RU of HLA-A2 Tax complex, the second coated with ~1000 RU of HLA-A2 NY-ESO complex, and two blank flow cells coated only with streptavidin (see Figure 15).

The increased affinity of the mutated soluble A6 TCR made calculation of the kd for the interaction of this moiety with the HLA-A2 Tax complex difficult. However, the half-life ($t_{1/4}$) for the interaction was calculated to be 51.6 minutes (see Figure 16), which compares to a $t_{1/4}$ for the wild-type interaction of 7.2 seconds.

Example 11 – Production of vector encoding a soluble NY-ESO TCR containing a novel disulphide bond.

The β chain of the soluble A6 TCR prepared in Example 1 contains in the native sequence a BglII restriction site (AAGCTT) suitable for use as a ligation site.

PCR mutagenesis was carried as detailed below to introduce a BamH1 restriction site (GGATCC) into the α chain of soluble A6 TCR, 5' of the novel cysteine codon. The sequence described in Figure 2a was used as a template for this mutagenesis. The following primers were used:

```
                    |BamHI |
5'-ATATCCAGAACCCgGAtCCTGCCGTGTA-3'  (SEQ ID 135)
5'-TACACGGCAGGAaTCcGGGTTCTGGATAT-3'  (SEQ ID 136)
```

100 ng of plasmid was mixed with 5 µl 10 mM dNTP, 25 µl 10xPfu-buffer (Stratagene), 10 units Pfu polymerase (Stratagene) and the final volume was adjusted to 240 µl with H_2O. 48 µl of this mix was supplemented with primers diluted to give a final concentration of 0.2 µM in 50 µl final reaction volume. After an initial denaturation step of 30 seconds at 95°C, the reaction mixture was subjected to 15 rounds of denaturation (95°C, 30 sec.), annealing (55°C, 60 sec.), and elongation (73°C, 8 min.) in a Hybaid PCR express PCR machine. The product was then digested for 5 hours at 37°C with 10 units of DpnI restriction enzyme (New England Biolabs). 10 µl of the digested reaction was transformed into competent XL1-Blue bacteria and grown for 18 hours at 37°C. A single colony was picked and grown over night in 5 ml TYP + ampicillin (16 g/l Bacto-Tryptone, 16 g/l Yeast Extract, 5 g/l NaCl, 2.5 g/l K_2HPO_4, 100 mg/l Ampicillin). Plasmid DNA was purified on a Qiagen mini-prep column according to the manufacturer's instructions and the sequence was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University.

cDNA encoding NY-ESO TCR was isolated from T cells according to known techniques. cDNA encoding NY-ESO TCR was produced by treatment of the mRNA with reverse transcriptase.

In order to produce vectors encoding a soluble NY-ESO TCR incorporating a novel disulphide bond, A6 TCR plasmids containing the α chain BamHI and β chain BglII restriction sites were used as templates. The following primers were used:

```
                | NdeI |
5'-GGAGATATACATATGCAGGAGGTGACACAG -3' (SEQ ID 137)
5'-TACACGGCAGGATCCGGGTTCTGGATATT-3' (SEQ ID 138)
                | BamHI|

                |NdeI |
5'-GGAGATATACATATGGGTGTCACTCAGACC-3' (SEQ ID 139)
5'-CCCAAGCTTAGTCTGCTCTACCCCAGGCCTCGGC -3' (SEQ ID 140)
       |BglII|
```

NY-ESO TCR α and β-chain constructs were obtained by PCR cloning as follows. PCR reactions were performed using the primers as shown above, and templates containing the native NY-ESO TCR chains. The PCR products were restriction digested with the relevant restriction enzymes, and cloned into pGMT7 to obtain expression plasmids. The sequence of the plasmid inserts were confirmed by automated DNA sequencing. Figures 17a and 17b show the DNA sequence of the mutated NY-ESO TCR α and β chains respectively, and Figures 18a and 18b show the resulting amino acid sequences.

Example 12 - Construction of phage display vectors and cloning of DNA encoding NY-ESO TCR α and β chains into the phagemid vectors.

DNA encoding soluble NY-ESO TCR α and β chains incorporating novel cysteine codons to facilitate the formation of a non-native disulfide inter-chain bond, produced as described in Example 11 were incorporated into the phagemid vector pEX746 as follows.

The DNA encoding the two NY-ESO TCR chains were individually subjected to PCR in order to introduce cloning sites compatible with the pEX746 phagemid vector (containing DNA encoding A6 TCR clone 7) using the following primers:

For the NY-ESO TCR alpha chain

TRAV21
GCCGGCCATGGCCAAACAGGAGGTGACGCAGATTCCT **(SEQ ID 141)**

YOL6
CTTCTTAAAGAATTCTTAATTAACCTAGGTTATTAGGAACTTTCTGGGCTG
GGGAAG **(SEQ ID 142)**

For the NY-ESO TCR beta chain

TRBV6-1/2/3/5/6/7/8/9
TCACAGCGCGCAGGCTGGTGTCACTCAGACCCCAAA **(SEQ ID 143)**

RT1
CGAGAGCCCGTAGAACTGGACTTG **(SEQ ID 144)**

The molecular cloning methods for constructing the vectors are described in "Molecular cloning: A laboratory manual, by J. Sambrook and D. W. Russell". Primers listed in table-1 are used for construction of the vectors. A example of the PCR programme is 1 cycle of 94°C for 2 minutes, followed by 25 cycles of 94°C for 5 seconds, 53°C for 5 seconds and 72°C for 90 seconds, followed by 1 cycles of 72°C for 10 minutes, and then hold at 4°C. The Expand hifidelity Taq DNA polymerase is purchased from Roche.

DNA encoding the clone 7 A6 TCR β chain was removed from pEX746 by digestion with restrictions enzymes BssHII and BglII. The correspondingly digested PCR DNA

encoding the NY-ESO β chain was then substituted into the phagemid by ligation. The sequence of the cloning product was verified by automated sequencing.

Similarly, DNA encoding the clone 7 A6 TCR α chain was removed from pEX746 by digestion with restrictions enzymes NcoI and AvrII. The correspondingly digested PCR DNA encoding the NY-ESO α chain was then substituted into the phagemid already containing DNA encoding the NY-ESO TCR β chain by ligation. The sequence of the cloning product was verified by automated sequencing.
Figures 19a and 19b detail respectively the DNA and amino acid sequence of the NY-ESO TCR α and β chain as well as surrounding relevant sequence incorporated in the phagemid (pEX746:NY-ESO). The sequence preceeding the NcoI site is the same as pEX746.

Example 13 – Expression of fusions of bacterial coat protein and heterodimeric NY-ESO TCR in E. coli.

Phage particles displaying the heterodimeric NY-ESO TCR containing a non-native disulfide inter-chain bond were prepared using methods described previously for the generation of phage particles displaying antibody scFvs (Li *et al*, 2000, Journal of Immunological Methods **236:** 133-146) with the following modifications. *E. coli* TG 1 cells containing pEX746:NY-ESO phagemid (i.e. the phagemid encoding the soluble NY-ESO TCR α chain and an NY-ESO TCR β chain fused to the phage gIII protein produced as described in Example 12) were used to inoculate 10 ml of 2x TY (containing 100μg/ml of ampicillin and 2% glucose), and then the culture was incubated with shaking at 37°C overnight (16 hours). 50 μl of the overnight culture was used to inoculate 10 ml of 2x TY (containing 100μg/ml of ampicillin and 2% glucose), and then the culture was incubated with shaking at 37°C until OD_{600nm} = 0.8. HYPERPHAGE Helper phage was added to the culture to the final concentration of 5 X 10^9 pfu/ml. The culture was then incubated at 37°C stationary for thirty minutes and then with shaking at 200 rpm for further 30 minutes. The medium of above culture was then made up to 50 ml with 2x TY (containing 100μg/ml of ampicillin and 25 μg/ml of kanamycin), the culture was then incubated at 25°C with shaking at 250 rpm

for 36 to 48 hours. The culture was then centrifuged at 4°C for 30 minutes at 4000 rpm. The supernatant was filtrated through a 0.45 μm syringe filter and stored at 4°C for further concentration. The supernatant was then concentrated by PEG precipitation and re-suspended in PBS at 10% of the original stored volume.

Example 14 – Detection of functional heterodimeric NY-ESO TCR containing a non-native disulfide inter-chain bond on filamentous phage particles

The presence of functional (HLA-A2-NY-ESO binding) NY-ESO TCR displayed on the phage particles in the concentrated suspension prepared in Example 13 was detected using the phage ELISA methods described in Example 4. Figure 20 shows the specific binding of phage particles displaying the NY-ESO TCR to HLA-A2-NY-ESO in a phage ELISA assay.

Example 15 - Construction of plasmids for cellular expression of HLA-DRA genes.

DNA sequences encoding the extracellular portion of HLA-DRA chains are amplified from cDNA isolated from the blood of a healthy human subject, using the polymerase chain reaction (PCR), with synthetic DNA primer pairs that are designed to include a Bgl II restriction site.

PCR mutagenesis is then used to add DNA encoding the Fos leucine zipper to the 3' end of the amplified sequencence.

DNA manipulations and cloning described above are carried out as described in Sambrook, J *et al*, (1989). Molecular Cloning - A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press, USA.

Figure 21 provides the DNA sequence of the HLA-DR β chain ready for insertion into the bi-cistronic expression vector. This figure indicates the position of the codons encoding the Fos leucine zipper peptide and the biotinylation tag.

Amino acid numbering is based on the mouse sequence (Kabat, 1991, Sequences of Proteins of Immunological Interest, 5th edition, US Dept of Health & Human Services, Public Health Service, NIH, Bethesda, MD 1-1137)

This DNA sequence is then inserted into a bi-cistronic baculovirus vector pAcAB3 (See Figure 22 for the sequence of this vector) along with DNA encoding the corresponding Class II HLA β chain for expression in Sf9 insect cells. This vector can be used to express any Class II HLA-peptide complex in insect cells.

Example 16. Construction of plasmids for cellular expression of HLA-DRB wild type and mutant genes.

DNA sequences encoding the extracellular portion of HLA-DRB chains are amplified from cDNA isolated from the blood of a healthy human subject, using the polymerase chain reaction (PCR), with synthetic DNA primer pairs that are designed to include a BamH1 restriction site.

PCR mutagenesis is then used to add DNA encoding the Jun leucine zipper to the 3' end of the amplified sequence and DNA encoding the Flu HA peptide loaded by the HLA-DR1 molecule to the 5' end of the sequence.

DNA manipulations and cloning described above are carried out as described in Sambrook, J *et al*, (1989). Molecular Cloning - A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press, USA.

Figure 23 provides the DNA sequence of the HLA-DR β chain ready for insertion into the bi-cistronic expression vector. This figure indicates the position of the codons encoding the Jun leucine zipper peptide and the Flu HA peptide.

Amino acid numbering is based on the mouse sequence (Kabat, 1991, Sequences of Proteins of Immunological Interest, 5th edition, US Dept of Health & Human Services, Public Health Service, NIH, Bethesda, MD 1-1137)

This DNA sequence is then inserted into a bi-cistronic baculovirus vector pAcAB3 (See Figure 22 for the sequence of this vector) along with DNA encoding the corresponding Class II α chain for expression in Sf9 insect cells. This vector can be used to express any Class II HLA-peptide complex in insect cells.

Example 17 Expression and refolding of Class II HLA-DR1- Flu HA complexes

Class II MHC expression is carried out using the bi-cistronic expression vectors produced as described in Examples 15 and 16 containing the Class II HLA-DR1 α and β chains and the Flu HA peptide. The expression and purification methods used are as described in (Gauthier (1998) PNAS USA **95** p11828-11833). Briefly, soluble HLA-DR1 is expressed in the baculovirus system by replacing the hydrophobic transmembrane regions and cytoplasmic segments of DR α and β chains with leucine zipper dimerization domains from the transcription factors Fos and Jun. In the expression construct, the required Class MHC-loaded Flu HA peptide sequence is covalently linked to the N terminus of the mature DR β chain and the DR α chain contains a biotinylation tag sequence to facilitate bifunctional ligand formation utilizing the biotin / strepavidin multimerisation methodology. The recombinant protein is secreted by Sf9 cells infected with the recombinant baculovirus, and purified by affinity chromatography. The protein is further purified by anion-exchange HPLC.

Example 18 –Construction of Class I soluble peptide-HLA molecules

In order to investigate further the specificity of the high affinity A6 TCR clone 134 the following soluble class I peptide-HLA molecules were produced:

HLA-A2 – peptide (LLGRNSFEV) **(SEQ ID 23)**
HLA-A2 – peptide (KLVALGINAV) **(SEQ ID 24)**

HLA-A2 – peptide (LLGDLFGV) **(SEQ ID 25)**
HLA-B8 - peptide (FLRGRAYGL) **(SEQ ID 26)**
HLA-B27 – peptide (HRCQAIRKK) **(SEQ ID 27)**
HLA - Cw6 – peptide (YRSGIIAVV) **(SEQ ID 28)**
HLA-A24 – peptide (VYGFVRACL) **(SEQ ID 29)**
HLA-A2 – peptide (ILAKFLHWL) **(SEQ ID 30)**
HLA-A2 – peptide (LTLGEFLKL) **(SEQ ID 31)**
HLA-A2 – peptide (GILGFVFTL) **(SEQ ID 33)**
HLA-A2 – peptide (SLYNTVATL) **(SEQ ID 34)**

These soluble peptide-HLAs were produced using the methods described in Example 10.

Example 19 – BIAcore surface plasmon resonance measurement of the specificity of Clone 134 high affinity A6 TCR binding to peptide-HLA.

A surface plasmon resonance biosensor (BIAcore 3000™) was used to analyse the binding specificitiy of the high affinity clone 134 A6 TCR. (See Figures 15a & 15b for the full DNA and amino acid sequences of the mutated TCR β chain respectively) This was carried out using the Class II HLA-DR1-peptide, produced as described in Examples 15 –17, and the Class I peptide-HLA complexes listed in Example 18, produced using the methods detailed in Example 10. The following table lists the peptide-HLA complexes utilised:

1. HLA-A2 – peptide (LLGRNSFEV) **(SEQ ID 23)**
2. HLA-A2 – peptide (KLVALGINAV) **(SEQ ID 24)**
3. HLA-A2 – peptide (LLGDLFGV) **(SEQ ID 25)**
4. HLA-B8 - peptide (FLRGRAYGL) **(SEQ ID 26)**
5. HLA-B27 – peptide (HRCQAIRKK) **(SEQ ID 27)**
6. HLA - Cw6 – peptide (YRSGIIAVV) **(SEQ ID 28)**
7. HLA-A24 – peptide (VYGFVRACL) **(SEQ ID 29)**

8. HLA-A2 – peptide (ILAKFLHWL) **(SEQ ID 30)**
9. HLA-A2 – peptide (LTLGEFLKL) **(SEQ ID 31)**
10. HLA-DR1- peptide (PKYVKQNTLKLA) **(SEQ ID 32)**
11. HLA-A2 – peptide (GILGFVFTL) **(SEQ ID 33)**
12. HLA-A2 – peptide (SLYNTVATL) **(SEQ ID 34)**

The above peptide HLAs were immobilised to streptavidin-coated binding surfaces in of the flow cells of a BIAcore 3000™ in a semi-oriented fashion.

The BIAcore 3000™ allows testing of the binding of the soluble T-cell receptor to up to four different pMHC (immobilised on separate flow cells) simultaneously. For this experiment three different HLA-peptides were immobilised in flowcells 2-4 and flowcell 1 was left blank as a control. Manual injection of HLA-peptide complexes allowed the precise level of immobilised molecules to be manipulated.

After the ability of the high affinity A6 TCR clone 134 to bind to the first 3 HLA-peptide complexes in the above list had been assessed the next three were immobilised onto these flowcells directly on top of the previous ones. This process was continued until the binding of the high affinity A6 TCR clone 134 to all 12 HLA-peptide complexes had been assessed.

Ten injections of 5 µl of the high affinity A6 TCR clone 134 were passed over each flowcell at at 5 µl/min at concentrations ranging from 4.1 ng/ml to 2.1 mg/ml. (See Figures 24-28)

As a final control the high affinity A6 TCR clone 134 was passed over a flowcell containing immobilised HLA-A2 Tax (LLFGYPVYV)**(SEQ ID 21)**, the cognate ligand for this TCR.

Specific binding of the high affinity A6 TCR clone 134 was only noted to its cognate ligand. (HLA-A2 Tax (LLFGYPVYV) **(SEQ ID 21))** These data further demonstrate the specificity of the high affinity A6 TCR clone 134. (See Figures 24-28)

Example 20 – Mutagenesis of NY-ESO TCR CDR3 regions

The CDR3 regions of the NY-ESO TCR were targeted for the introduction of mutations to investigate the possibility of generating high affinity mutants. This was achieved using NY-ESO TCR-specific PCR primers in combination with methods substanitially the same as those detailed in Example 7.

Example 21 – Isolation of high affinity A6 TCR mutants

The isolation of high affinity NY-ESO TCR mutants was carried using out the first of the two methods described in Example 8.

A single high affinity NY-ESO TCR mutant was identified.

Example 22 – Production of soluble high affinity heterodimeric NY-ESO TCR with non-native disulfide bond between constant regions, containing variable region mutations

Phagemid DNA encoding the high affinity NY-ESO TCR mutant identified in Example 21 was isolated from the relevant *E.coli* cells using a Mini-Prep kit (Quiagen, UK)

PCR amplification using the phagemid DNA as a target and the following primers were used to amplify the mutated soluble NY-ESO TCR β chain variable region DNA sequence.

NY-ESO beta chain forward primer
Tctctcattaatgaatgctggtgtcactcagacccc **(SEQ ID 145)**
(AseI restriction site is underlined)

Universal beta chain reverse primer
Tagaaaccggtggccaggcacaccagtgtgg **(SEQ ID 146)**
(AgeI restriction site is underlined)

The PCR product was then digested with AgeI/AseI and cloned into pEX821 (Produced as described in Example 11) cut with Nde/AgeI.

The mutated NY-ESO TCR β chain DNA sequence amplified as described above, and the NY-ESO TCR α chain produced as described in Example 11 were then used to produce a soluble high affinity NY-ESO TCR as described in WO 03/020763. Briefly, the two chains are expressed as inclusion bodies in separate *E.coli* cultures. The inclusion bodies are then isolated, de-natured and re-folded together *in vitro*.

Example 23 – BIAcore surface plasmon resonance characterisation of a high affinity NY-ESO TCR binding to HLA-A2 NY-ESO.

A surface plasmon resonance biosensor (Biacore 3000™) was used to analyse the binding of the high affinity NY-ESO TCR to the HLA-A2 NY-ESO ligand. This was facilitated by producing pMHC complexes (as descrtibed in Example 10) which were immobilised to a streptavidin-coated binding surface in a semi-oriented fashion, allowing efficient testing of the binding of a soluble T-cell receptor to up to four different pMHC (immobilised on separate flow cells) simultaneously. Manual injection of HLA complex allows the precise level of immobilised class I molecules to be manipulated easily.

The interactions between the high affinity NY-ESO TCR containing a novel inter-chain bond and the HLA-A2 NY-ESO complex or an irrelevant HLA-A2 Tax combination, the production of which is described in Example 10, were analysed on a Biacore 3000™ surface plasmon resonance (SPR) biosensor, again as described in Example 10.

The kd for the interaction of the soluble high affinity NY-ESO with the HLA-A2 NY-ESO was calculated to be 4.1 μm, (See Figures 29a and 29b) which compares to a kd of 15.7 μm for the wild-type interaction. (See Figures 30a and 30b)

Example 24 – Production and testing of further High Affinity A6 TCRs

Soluble TCRs containing the following mutations corresponding to those identified in clones 89, 1, 111 and 71 (see Example 8) were produced using the methods detailed in Example 9. The binding of these soluble TCRs to HLA-A2 Tax was then assessed using the Biacore assay detailed in Example 10.

A6 TCR β chain mutants

Clone	CDR3 sequence
Wild Type	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCAGAGCAGTAG **(SEQ ID 83)** A S R P G L A G G R P E Q Y **(SEQ ID 84)**
89	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCAGAGGATTAG **(SEQ ID 91)** A S R P G L A G G R P E **D** X **(SEQ ID 92)**
1	GCCTCGAGGCCGGGGCTGGTTCCGGGGCGACCAGAGCAGTAG **(SEQ ID 97)** A S R P G L **V** **P** G R P E Q X **(SEQ ID 98)**
111	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCACATCCGTAG **(SEQ ID 101)** A S R P G L A G G R P **H** **P** X **(SEQ ID 102)**

A6 TCR α chain mutant

Clone	CDR3
Wild Type	GCCGTTACAACTGACAGCTGGGGGAAGCTTCAG **(SEQ ID 107)** A V T T D S W G K L Q **(SEQ ID 108)**
71	GCCGTTACAACTGACAGCTGGGGGGAGCTTCAT **(SEQ ID 117)** A V T T D S W G **E** L **H** **(SEQ ID 118)**

Combined mutations used as a basis for the production of mutated soluble A6 TCRs:

Clone 89 mutations + Clone 134 mutations

Clone 71 mutations + Clone 134 mutations

Clone 71 mutations + Clone 89 mutations

Clone 1 mutations + βG102→A mutation

Results:

The following table compares the HLA-A2 Tax affinity of the above soluble mutated A6 TCRs to that obtained using a soluble A6 TCR containing unmutated variable regions. Note that the affinity of the highest affinity mutants is expressed as the half-life for the interaction. ($T_{1/2}$) These soluble mutant A6 TCRs exhibited higher affinity for HLA-A2 Tax than the unmutated soluble A6 TCR as demonstrated by their lower kd or longer $T_{1/2}$ for the interaction.

Figures 31 – 37 show the Biacore traces used to calculate the affinity for HLA-A2 Tax of these soluble mutated TCRs. Figures 38a-e show the amino acid sequence of the mutated A6 TCR chains.

A6 TCR	Kd (μM)	$T_{1/2}$ (Secs)
Wild-type	1.9	7
Clone 1		810
Clone 89	0.41	
Clone 111	1.18	
Clone 71	1.37	
Clone 89 + Clone 134 mutations		114 (phase 1) 4500 (phase 2)
Clone 71 + Clone 134 mutations		882
Clone 71 + Clone 89 mutations	0.35	
Clone 1 + βG102→A mutation		738

Example 25 – Cell staining using High affinity A6 TCR tetramers and monomers

T2 antigen presenting cells were incubated with β2m (3μg/ml) pulsed with Tax peptide at a range of concentrations (10^{-5} – 10^{-9}M) for 90 minutes at 37°C. Controls, also using T2 cells incubated with β2m (3μg/ml), were pulsed with 10^{-5}M Flu peptide or incubated without peptide (unpulsed). After pulsing the cells were washed in serum-free RPMI and 2 x 10^5 cells were incubated with either strepavidin-linked high affinity Clone 134 A6 TCR tetramer labelled with phycoerythyrin (PE). (Molecular probes, The Netherlands) (10μg/ml) or high affinity Clone 134 A6 TCR monomers labelled with Alexa 488 (Molecular probes, The Netherlands) for 10 minutes at room temperature. After washing the cells, the binding of the labelled TCR tetramers and monomers was examined by flow cytometry using a FACSVantage SE (Becton Dickinson).

Results

As illustrated in Figure 39a specific staining of T2 cells by high affinityA6 TCR tetramers could be observed at Tax peptide concentrations of down to 10-9 M.

As illustrated in Figure 39b specific staining of T2 cells by high affinityA6 TCR monomers could be observed at Tax peptide concentrations of down to 10-8 M.